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MUSEUM TECHNIQUES : A REVIEW

BY

R. J. V. PULVERTAFT

From the Westminster Hospital School of Medicine, London

(RECEIVED FOR PUBLICATION DECEMBER 15, 1949)

The preservation of pathological material has never been of greater importance than at present, when the introduction of new, successful methods of therapy is changing the picture of disease out of all recognition. It is interesting to walk through any medical museum established a hundred years and to note how many specimens relate to conditions which are either never or rarely seen at necropsy to-day or which, if encountered, are greatly modified by treatment. On the other hand, surgical treatment, by prolonging life, often permits of conditions not previously encountered; it is a matter of interesting conjecture to consider what disorders may in future manifest themselves in increasing numbers. Obvious examples are the degenerative conditions of old age, as the treatment of acute infections becomes progressively more effective. Pathological museums are in part historical, representing the pioneer work of diagnosticians and therapists; in part they are becoming museums in the generally accepted sense of the word, presenting records of past states not now encountered, or conditions of great rarity; and finally they provide the student with the basic material of his current teaching. But no one can prophesy when the last class will be converted almost overnight into one of the former.

Collection of Material

The great majority of museum specimens are collected from teaching hospitals. This is a great pity, since by their nature such specimens cover only a part of human disease, excluding, for example, chronic conditions, and in any case only a small part of the available material passes through the teaching hospitals. It is most desirable that a centralized clearing station for specimens should be established.

From every point of view, and in particular to ensure the best museum specimens, necropsies should be neither hurried nor perfunctory. They should be performed at leisure before a limited audience, and the demonstration of material should be the subject of a separate session. Necropsies should of course be performed as soon as possible after death, but in many cases, particularly in order to preserve the stomach, intestinal tract, or brain, 4% formol saline should be injected into the peritoneal cavity, the carotid arteries, or stomach as soon as possible after death.

One of the commonest causes of inferior specimens is contact with tap water. The resultant haemolysis greatly reduces their value. Specimens should be washed only with saline, and should be kept in saline while awaiting demonstration as

drying again ruins the surface appearances, but as autolysis quickly sets in they should not remain in saline for more than two hours.

Material for microscopical section should, if possible, be selected at the time of mounting the specimen, and it is often most desirable not to open hollow viscera or to dissect specimens until fixation is complete.

It is essential that the cut surface of a specimen should be flat. In many cases this can be ensured by passing a glass rod into a structure such as a large bronchus and cutting along the rod with a large broad knife. Many special methods have been devised. Lundy and Camp (1939) describe a technique for large anatomical specimens. Anderson (1929) has designed an apparatus comprising a flat board with glass rods, which is particularly valuable for brain slices. Gough and Wentworth (1949) have been particularly successful with preparations of lungs in cases of pneumoconiosis, when the lungs are inflated and immersed in fixative; thin slices are then cut with a knife by hand, and impregnated with increasing strengths of gelatine. After final embedding in formol gelatine, sections of 600 μ thickness are cut on a large microtome and the sections are laid on sheets of "perspex." Sheets of Whatman's No. 1 filter paper are squeezed over the surface and left to dry; the sections adhere to the paper, and can be peeled off. The specimens can then be filed in book form.

FIG. 1.—Demonstration of bronchial tree by plastics. (Photograph kindly lent by Dr. A. A. Liebow, U.S.A.)

Vowles (1942) describes a cheap method of mounting thin slices of organs in pure Trinidad Lake asphalt. Hall (1944) uses a rotary meat slicer, and mounts specimens stained or unstained between sheets of glass sealed with a paraffin wax mixture. Freezing and cutting with a hand-saw is useful, especially for bones prepared with soft tissues. A method has been described by Baker (1940).

Injection techniques, especially for the vascular system, are often necessary at the necropsy. Narat, Loef, and Narat (1936) have elaborated a vinyl resin technique, permitting of multicoloured effects, followed by corrosion with fuming hydrochloric acid; Leibow (1947) and Hill (1937) also suggested the use of latex

(Fig. 1). We have followed the method of Lieb (1940), using liquid "neoprene," with excellent results. Further details of this valuable technique are given by Duff and More (1944), and for the coronary system by Smith and Henry (1945).

Fixation

There are certain important general principles of fixation. Specimens containing bile or stained by bile must be fixed and stored apart from others, as they will stain them. Specimens undergoing fixation must not touch other specimens, or the sides of jars; they must either lie on washed fluffless lint or be suspended by linen thread. Specimens may be pressed out on cork, or stretched on glass or metal frames, but this causes distortion; it is often preferable to fill hollow viscera with cotton-wool soaked in fixative, tie off at both ends, and immerse them in a fixing tank. Unopened cystic cavities should be injected with fixative; if opened they should be packed with cotton-wool. Certain solid viscera should be fixed by vascular injection—e.g., the brain through the basilar artery. Dukes and McDonnell (1938) give particulars for the treatment of renal specimens, which should never be cut before fixation. Dukes and Bussey (1936) have described a method of preparing specimens of intestinal tumours which has become widely used and should be consulted by anyone who may not be familiar with it. The lungs and limbs are particularly suitable for fixation by vascular injection. The spleen should never be cut before fixation, but unfortunately, even after vascular injection, patchy fixation is often a nuisance. For diagnostic purposes specimens must be taken at the time of necropsy.

The preservation of the colour and form of specimens depends entirely on the fluids used to fix and to mount them. The original method of Kaiserling (1897) is still widely used. Three solutions are employed, with the following formulae:—

Kaiserling No. I		
	Formalin (40%)	400 ml.
	Pot. nitrate	30 g.
	Pot. acetate	60 g.
	Tap water	2,000 ml.
Kaiserling No. II		
	Ethyl alcohol	80%
Kaiserling No. III		
	Glycerine	500 ml.
	Arsenious acid 1%	200 ml.
	Pot. acetate	250 g.
	Thymol	2.5 g.

There are several important details to be observed if this method is employed. Fixation in formalin is almost universal, but the danger of formalin to technicians and pathologists is not adequately recognized. Not only does it cause dermatitis, but its effect on the nasal mucosa is also serious, and sinusitis is a common result. A museum mounting room must be thoroughly ventilated and an exhaust fan fitted.

Specimens should be fixed in Solution No. I for periods up to two weeks depending on their size, and larger specimens should always be injected. In this solution the colour contrasts disappear, and are to some extent restored by the

ethyl alcohol, a rather expensive reagent. Specimens should remain in alcohol for periods of up to one hour, but must be carefully watched to ensure that they are removed when the optimum stage is reached; if kept for longer periods the colour fades and cannot be restored.

Solution No. III is the mounting fluid, containing arsenious acid and thymol to inhibit moulds, and glycerine, again an expensive reagent, for its effect on the refractive index. It is difficult to get the arsenious acid into solution, but good results are obtained by making up 1% arsenic trioxide in water and steaming the solution for six hours in a steam sterilizer. The thymol is ground up and floated on the surface of the fluid (Kaiserling, 1897). In later modifications the glycerine content was raised to 30%, and 0.5% formalin was substituted for the arsenic and the thymol.

The colour of specimens in Kaiserling's fluid leaves much to be desired and fading sets in rather soon. Jores (1913) modified the method, which was further altered by Klotz and Maclachlan (1915), and this latest modification is intended whenever Kaiserling is referred to in this article.

Solution I (Colour Fixation Fluid)

Chloral hydrate	50 g.
Carlsbad salts (artificial)	50 g.
Formalin	100 ml.
Tap water	1,000 ml.

The composition of the Carlsbad salts is

Sod. sulphate	22 g.
Sod. bicarbonate	20 g.
Sod. chloride	18 g.
Pot. nitrate	38 g.
Pot. sulphate	2 g.

After fixation for periods of up to six weeks specimens are washing in running water for 12 hours and then mounted in Solution No. II.

Solution II

Pot. acetate	300 g.
Glycerine	600 ml.
Dist. water	1,000 ml.

The advantages of this method were combined by Aegerter (1941) in the following formula:—

Colour Fixation Fluid

Sod. chloride	140 g.
Sod. bicarb.	80 g.
Chloral hydrate	625 g.
Formalin (40%)	512 ml.
Dist. water to	20 litres

After immersion in this fluid for up to 20 hours specimens are placed in the original Kaiserling I solution for from one to five days without washing and then, again without washing, mounted in a modified Kaiserling III solution

Pot. acetate	2,000 g.
Glycerine	4,000 ml.
Thymol	5 g.
Distilled water	20 litres

A very large number of other fixatives and mounting media have been described. For special purposes special fixatives are necessary. The method used for most purposes at the Westminster Medical School is based on a personal observation (Pulvertaft, 1936) that the colour of faded specimens of chloroma could be restored by the addition of sodium hydrosulphite to the medium; as a result all specimens mounted during the last 13 years have been so preserved. Further papers on this method have been provided by Wentworth (1938, 1942, 1947).

The method in use at the Westminster Medical School is as follows. Specimens are fixed in Kaiserling Solution No. I, and after fixation transferred to fresh Kaiserling Solution No. I as modified for storing. For mounting they are transferred to Kaiserling Solution No. III.

Glycerine	30%	adjust to *p*H 8
Sod. acetate (B.P.)	10%	
Formalin	0.5%	

If the solution is not crystal clear, impurities in the sod. acetate are usually to blame. Such solutions should be filtered through paper pulp under negative pressure. Immediately before sealing 0.4% sod. hydrosulphite is added. It is important that specimens should not be rinsed in tap water even after fixation.

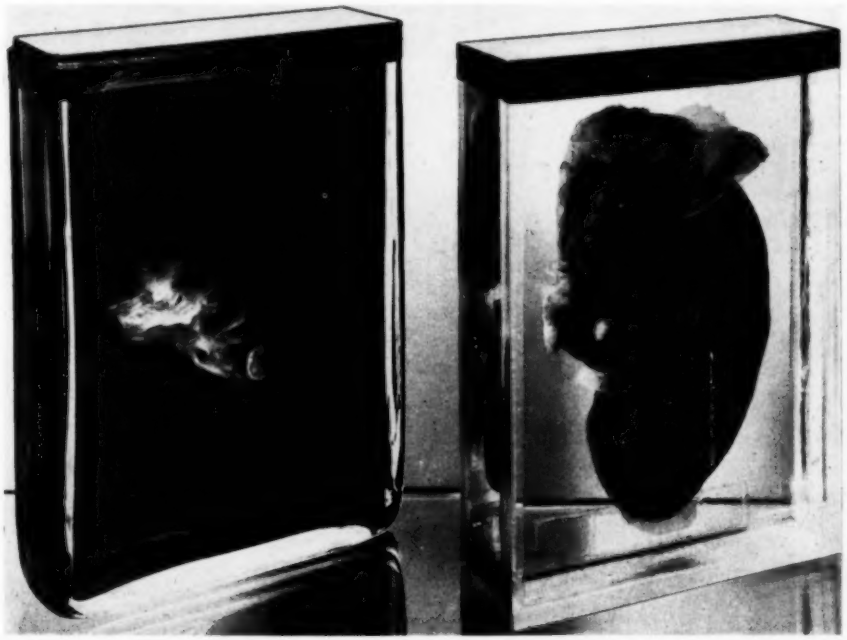


FIG. 2.—Two halves of the same kidney. The specimen on the left has been washed in running water for 12 hours, that on the right in normal saline.

Experiments performed by Dr. A. D. Morgan show that haemolysis later sets in (Fig. 2) if there is much blood when specimens are washed in running water. Saline or Kaiserling Solution No. I may be used for rinsing. The amount of hydrosulphite should not normally exceed 0.4%. If colour restoration must be rapid, 0.6% may be added, but this is to be avoided, as a white precipitate may form.

Jars must be sealed immediately after mounting, and of course the lid must not be perforated, a practice necessary with large glass jars to prevent cracking on cooling in winter but quite unnecessary with "perspex" jars. Unsatisfactory results are due to inadequate fixation, washing with tap water after fixation, excess hydrosulphite, delayed sealing of the jars, acidity of the mounting fluid (whose *p*H should be 8), or the use of stale formalin containing *para*-formaldehyde as a white precipitate. Fragile specimens, such as embryos, shrink in this mounting medium owing to osmosis, which may be prevented by lowering the glycerine content and by injecting gelatine into the cranial cavity. Wentworth (1942) omits glycerine in the mounting medium. This is an economy, but reduces the refractive index of the medium and therefore causes a loss of brilliancy.

Carbon monoxide has also been employed as a colour-retaining agent. Schultz (1931) introduced the technique, which gives brilliant colour contrast, but entails the risks of poisoning and explosion. These may be avoided by the technique of Robertson and Lundquist (1934). The method is also described by Lewis and Gaines (1936). Kaiserling I solution is used while the specimens are being gassed, and final mounting is in Kaiserling III.

Both the hydrosulphite and CO methods produce brilliant colour contrasts; by the hydrosulphite method colours are retained with little fading for 13 years at least; by the CO method no change has been noted to date. However, the colours are not natural in either case and for this reason some curators dislike the methods. There is probably no ideal mounting medium, as the taste and objectives of curators differ.

Maceration of Bones

The maceration technique is particularly useful in showing osteogenic sarcomata, but it has many other uses. Methods are reviewed by Wagoner and Nuckols (1935). For delicate specimens prolonged putrefaction gives the best results. Quick results for gross specimens are given by autoclaving them for 10 minutes at 10 lb. pressure with N/1 NaOH. As a routine, maceration in N/4 NaOH at a temperature of 90° C. gives excellent results. During maceration the specimen is removed at intervals, and, after washing in a stream of water, portions of soft tissue are removed with forceps. When all the soft tissues have been removed specimens are dried in an incubator, and grease is removed by immersion in chloroform for three hours. They are then bleached by H_2O_2, and are mounted dry in "perspex" jars (Figs. 3 and 4).

Storage of Specimens before Mounting

Labels are readily made from old *x*-ray films by removing the emulsion in hot water. They are then immersed in glacial acetic acid for 10 seconds, and placed in a solution of 5 g. sod. carbonate in a gallon of water until they become

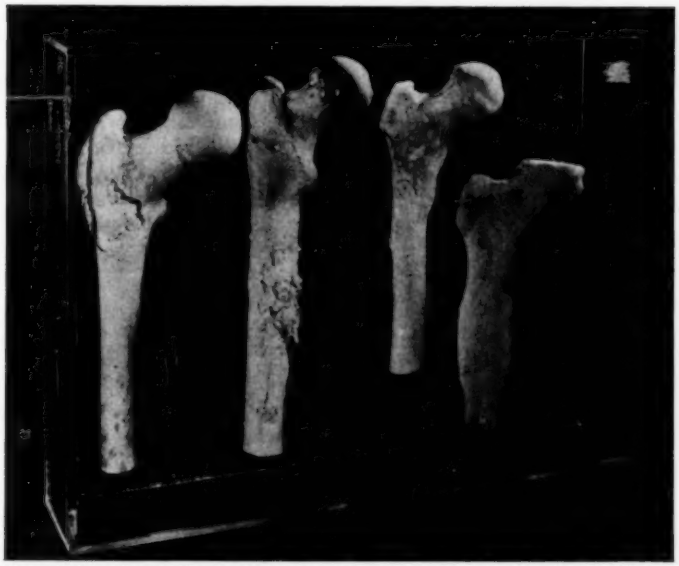


FIG. 3.—Macerated specimens of osteogenic sarcomata mounted on a centre-plate in a " perspex " box.

FIG. 4.—Macerated specimen of normal mandible demonstrating the use of " perspex " supports which have been cemented into position.

opalescent. They are next washed and dried, and a hole is punched to take a string. Identification details are written in indian ink. Immersion in acetone for a few seconds makes the label permanent. Two labels are prepared, and one is attached directly to a corner of a gauze square in which the specimen is wrapped by tying the corners of the square together. The other label is attached to a corner of the gauze square by a length of linen thread. Specimens are kept in Kaiserling I solution while awaiting mounting. Earthenware tanks of 25-gallon capacity measuring 34 in. × 22 in. × 19 in. may be procured, and surplus aluminium containers, unaffected by Kaiserling solution, can also be obtained. The tanks

FIG. 5.—Porcelain storage tank, showing method of keeping labels in order with wood strips. Necessary materials for wrapping and labelling specimens are displayed on the teak cover. One specimen is shown wrapped and labelled ready for storage.

are covered, preferably with teak. Strips of wood of the same length as the sides of the tank are provided and saw cuts $\frac{1}{8}$ in. deep are made at intervals of 1 in. Through these notches the linen threads with attached labels hang, and thus specimens may quickly be selected (Fig. 5).

Treatment of Specimens before Mounting

Cavities, such as spermatoceles, should be filled with gelatine prepared as follows:

Using a reflux condenser 20 g. arsenious acid is boiled in 1 litre of water for two hours in a fume chamber. The solution is cooled and 120 g. of gelatine previously soaked in warm water added, excess water being removed by squeezing. It is then filtered through sand and paper pulp, and 10% glycerine and 0.5% Victoria blue added

drop by drop until the bulk is faintly blue; the gelatine should still appear colourless in a test-tube. The prepared gelatine is stored in the dark, and before use 0.4% formalin is added.

Anatomical details may be marked by "perspex" strips cut from $\frac{1}{16}$ in. sheets ground to the desired shape by a carborundum wheel. "Perspex" rods and arrows can also be made with ease.

Friable specimens may be coated with gelatine as suggested by Wentworth (1947).

The bile discoloration from livers and gall-bladders can to a certain extent be prevented by soaking specimens in a saturated solution of calcium chloride for 24–48 hours before mounting.

Mounting in Jars

Plastic museum jars are now almost universally used in Britain, although in the U.S.A. watch-glass techniques are often preferred (Davis, 1942; Brown and Schroeder, 1937). Plastic watch-glasses can also be used (Larson and Levin, 1940). The advantages of plastic jars are numerous. Their inner and outer surfaces are parallel and free from ridges and there is no distortion. Their refractive index is 1.49, and light transmission is up to 92%. (The properties of plastic jars are fully discussed by Bartoe, 1940, and their use in museum work is reviewed by Kramer, 1940.) Plastic jars will stand severe strain. It is well known that large glass jars break with temperature changes if sealed, and it is not possible to use the hydrosulphite method with large specimens in glass jars. Plastic jars can be dropped 6 ft. without breaking. In a museum of 2,500 specimens an average of five breakages a week was found in glass-mounted specimens.

Fluids can be changed by drilling two holes in the base with a $\frac{1}{16}$ in. drill, the holes being later filled with "perspex" soaked in chloroform; cement is finally applied.

The plastic jars scratch readily, although it is found that specimens in constant use have not needed attention for three years. Scratches are easily removed by metal polish.

Apparatus for Making Plastic Jars.—Boxes or jars can be made with no more than a tenon saw and sand-paper, but the following apparatus is desirable if numbers are to be made.

(1) A 6 in. bench grinder. This is an electric motor with spindles at each end, one end being fitted with a carborundum wheel, the other with a calico mop for polishing (cost, £15).

(2) A band facing machine, 36 in × 4 in. This is a motor-driven sand-papering machine, with the advantages of precision and speed (cost, £24).

(3) A circular saw ("sylvan" No. 2). This has a rise-and-fall 8 in. blade, 16 teeth to the inch, with a slight set to avoid clogging. It has horizontal and vertical guides (cost, £40).

In making jars the sides must be cut absolutely square with finely ground edges to prevent bubbles. Welding is effected by immersion of the edges in chloroform or ethylene dichloride, or by "perspex" cement, No. 2 being fast-setting and No. 6 slow-setting. The front of the jar is cemented to the sides, supported in a former, the back is next cemented on, followed by the bottom and the top.

An alternative method of making jars has been devised by Professor Duguid, by whose courtesy the following method is presented.

A heating appliance for bending "perspex" is needed. This may be in the shape of two ½ in. copper pipes accurately fitted with a right angle guide so as to form a set square. Jets of steam are blown through the pipes and the strip of "perspex" laid between them for 12–15 minutes. It can then be easily bent and held at a right angle until cooled under water. A more convenient apparatus consists of two copper rod heating elements connected with a transformer so as to pass a high current of 700 amps at ½ volt. This heats the "perspex" to flexibility in about five minutes.

Method of Making Plastic Jars.—A rectangular strip of ⅛ in. "perspex" is bent to right angles at two points so as to form the top and sides of the jar, and the edges are accurately trimmed with a circular saw. One edge is soaked in ethylene dichloride for 10 minutes. This can be done by propping the strip on three pieces of fine wire or nylon thread laid on a glass slab and running ethylene dichloride around the lower edge so as to form a pool of the fluid. When the edge is softened a roughly cut sheet of ⅛ in. "perspex" is applied to it so as to form one face of the jar, and gentle pressure applied by weights. In 10–15 minutes the joint is firm enough to permit manipulation, and the other face can be fixed by the same process. Weights up to 14 lb. should then be applied overnight. Next day with the circular saw the redundant parts of the facing sheets are cut off flush with the sides of the jar and the edges polished on the buffing machine. The bottom of the jar is also squared off with the circular saw so as to leave an even rim to which the base is fixed. A rectangular slab of ⅛ in. "perspex" is cut and polished to form a base, and a $\frac{1}{16}$ in. hole is bored in it through which the jar can finally be filled with mounting fluid. The specimen to be mounted is fixed to a $\frac{1}{16}$ in. "perspex" sheet, which is cut to fit firmly in the jar. The lower rim of the jar is softened by ethylene dichloride and the jar inverted so that the specimen can be quickly lowered into it. Sufficient mounting fluid to cover the specimen is poured in and the base is then laid in position and weights applied overnight. Next day the jar is completely filled with fluid through the hole in the base so that all air is

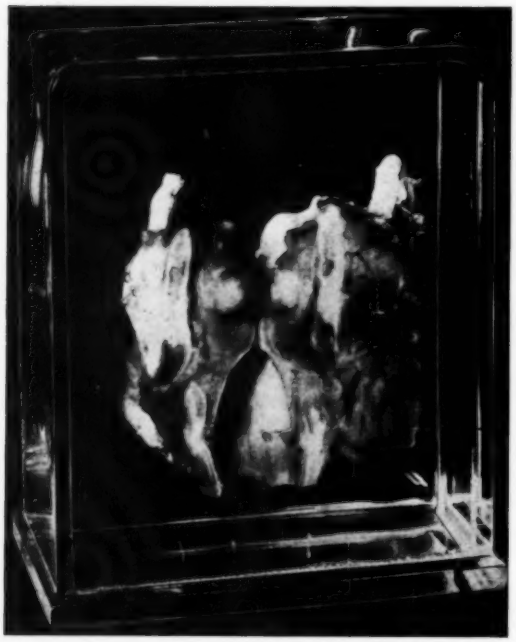


FIG. 6.—"Perspex" museum jar made by the method originated by Professor Duguid. The bending of the corners was effected with the flame from a pasteur pipette. The "perspex" stop to keep the centre-plate in position can be seen on the inside of the base of the jar; a similar one is cemented to the top.

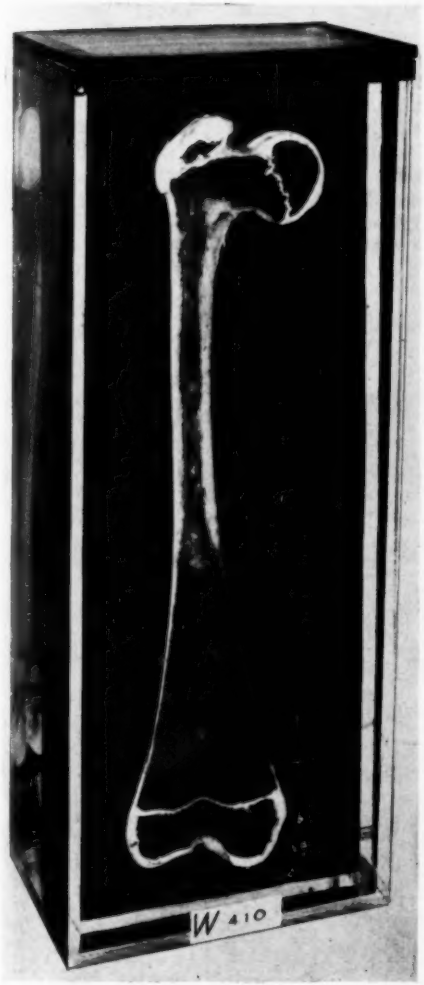
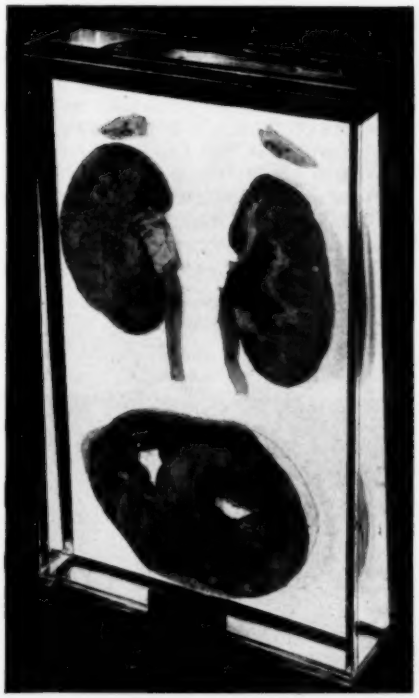
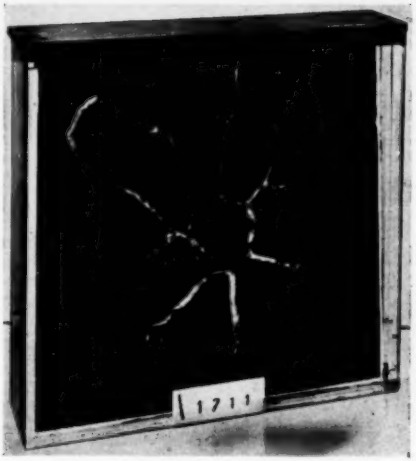


FIG. 7.





FIGS. 8 and 9.

FIG. 7.—The femur from a case of Gaucher's disease mounted on a black "perspex" centre-plate to contrast with the white bone and bright red bone marrow.

FIG. 8.—Kidneys, suprarenals, and heart from a case of acute malignant hypertension, mounted on a white centre-plate to contrast with the bright colour of the specimen.

FIG. 9.—An aneurysm of the circle of Willis fixed in position on a centre-plate of black "perspex" with a thin layer of gelatine. The jar with centre-plate in position was filled with mounting fluid before sealing.

expelled, and the hole finally plugged with a peg of "perspex" soaked in ethylene dichloride.

There are several modifications of the method of heating the "perspex" strip for bending. One consists of heating a length (6 in.) of copper tubing in a batswing burner flame, having previously marked with a scribe the place at which the bend is required. When the copper tube is sufficiently hot the scribe mark is laid along it; this will burn away the mark from the "perspex," also heating it right through. When it is pliable the strip is bent into a right angle, using a framework to ensure that it is square, and held or clamped in this position until cold.

Another simple method is to use a finely drawn pasteur pipette as a gas jet and draw it across the "perspex" strip on both sides at the desired place. When it is pliable it is treated as described above (Fig. 6). The principle is to heat as narrow a strip across the "perspex" as is consistent with bending.

Method of Mounting.—The specimen should be measured, and $\frac{1}{2}$ in. clearance allowed at the sides and 1 in. at the top and bottom.

FIG. 10.—Calculi mounted by the method described by Culling.

The specimen should be mounted on a centre-plate; if more than one specimen is mounted in the same container opaque "perspex" should be used (Figs. 7, 8, 9). (This practice is often invaluable for demonstrating allied lesions.) The specimen is laid on the centre-plate, and scratches made to indicate the position of ties. Two $\frac{1}{16}$ in. holes are drilled with a dentist's drill about $\frac{1}{8}$ in. apart and the specimens stitched on with nylon thread, which is practically invisible in the fluid. Nylon is difficult to tie, and a surgeon's knot is necessary. The tie should be on the face of the specimen. Centre-plates can be bent to any required shape by heating in an oven to 78° C. if plasticized, or 105° C. if unplasticized. They are then immersed in cold water. Over-heating causes bubbling. The centre-plates are held in position by "perspex" strips cemented in place (Fig. 6).

When the specimen has been placed in the jar mounting medium and hydrosulphite is run in to within $\frac{1}{4}$ in. of the top, care being taken that trapped bubbles are released.

" Perspex " (5%) in chloroform or " perspex " cement No. 2 or 6 is applied to the top edges with a pasteur pipette, and after 30 seconds the lid is applied lightly; if any " perspex " exudes it must immediately be removed. A light weight is then applied for one hour. The lid must have a hole bored in it to introduce the remaining mounting medium with which the jar is entirely filled. After 48 hours residual bubbles are removed. A " perspex " plug is made by cutting a pasteur pipette so that its internal diameter is slightly larger than that of the hole in the lid. A piece of " perspex " is heated until pliable, and the pipette is then used like a cork borer to remove a plug. This can be readily expressed from the pipette and naturally tapers; alternatively, $\frac{1}{8}$ in. diameter " perspex " rod is now available for this purpose. The plug is then tapped into position. A drop of cement may be added but is not usually necessary.

Calculi may be mounted by a method described by Culling (1949) (Fig. 10).

Macerated bones are mounted dry but need a firm support. Nylon " monomer " filament 252 is desirable for fixation on the centre-plate, and " perspex " cement should be applied to the ties and portions of the specimen touching the centre-plate (Figs. 3 and 4).

Membranes may be demonstrated with a " perspex " lens (Fig. 11).

FIG. 11.—Method of demonstrating a membrane by means of a " perspex " cup which when filled with fluid acts as a low power lens. (Photograph kindly lent by Mr. D. Martin, Hospital for Sick Children, Great Ormond Street, London.)

Gelatine Embedding.—This method was formerly much used with glass containers, as breakages were fewer and did not cause trouble from fluid loss. Colour preservation after treatment with Kaiserling Solutions I and II is good.

Gelatine is prepared as previously described in this paper, and formalin added just before use. With face uppermost the specimen is coated with gelatine, and when the gelatine has set it is placed face downwards in the jar. A piece of adhesive tape is fixed across the top of the jar and gelatine is later added to a depth of $\frac{1}{4}$ in. to hold the specimen in place. The technique up to this point may be used to hold delicate specimens in position with the hydrosulphite method (Fig. 9). After setting in a refrigerator the jar is filled with prepared gelatine, bubbles being burst with a hot needle (Sahasrabudhe, 1934). Water miscible plastics which set are also now obtainable.

Mounting in Solid " Perspex "

The ideal museum specimen should be mounted in a transparent, unbreakable solid mass, and should retain its natural colour and shape indefinitely. The embedding of biological material in transparent plastics is still in the experimental stage and the ideal plastic has yet to be discovered. Unless great care is taken to prevent an undue temperature being reached during polymerization, especially

when thick layers or blocks are being handled, internal stresses may result in damage to the specimens or in fissures in the plastic. This difficulty may be avoided by lowering the temperature for polymerization or reducing the quantities of catalyst and accelerator used which will slow the rate of polymerization. An early advance towards the ideal objective was made by Carroll and Neidhoefer (1936), who accidentally embedded specimens in celluloid, after immersing them in oil of wintergreen which dissolved the celluloid. Plastics would seem to be ideally suited to this technique, but many difficulties have been encountered, owing to bubble formation and clouding of the plastic; the relevant problems are discussed by Hibben (1937) and Kramer (1940), who remind us that the bee in amber is an early example of this technique.

A very elaborate method, with certain unique features, has been designed by Strumia and Hershey (1948). Specimens are not fixed or dehydrated by any of the traditional methods, but frozen to a temperature of −20° C. and then dehydrated from the frozen state by sublimation of water vapour. They are then impregnated with a liquid monomer, and subsequently polymerized with benzoyl peroxide. The colours are said to remain unchanged for at least five years, and their coloured illustrations show a remarkable degree of preservation of the shape, texture, and colour of a xanthoma. It is clear that a large initial outlay on equipment is necessary for this method.

Biologists will all agree that any method of by-passing the traditional methods of fixation and dehydration deserve serious consideration, for it is to these that we must ascribe the unlifelike appearance of tissues.

Three Alternative Methods for Opaque Specimens.—(1) A method supplied by I.C.I. Plastics, Ltd., is at present under trial, and the following materials are required:

Methyl methacrylate monomer
Benzoyl peroxide
Dibutyl phthalate
Sheet "perspex" $\frac{1}{16}$ in. or $\frac{1}{8}$ in. thickness
Caustic soda
"Perspex" cement No. 6

Washing of Monomer.—During manufacture the methyl methacrylate monomer is stabilized by the addition of hydro quinone and is then known as "kallodoc" liquid. Before thickening the stabilizer must be removed by washing with 5% caustic soda solution in the following manner. Equal quantities of monomer and caustic soda should be used, and washing is best carried out in a separation funnel, where the discoloured washings, which form a layer below the monomer, can easily be run off. Two washings with the 5% caustic soda solution are sufficient, and should be followed by washing with water, until the liquid run off is no longer alkaline (test with phenolphthalein). As before, the monomer forms the top layer in the separating funnel. The methyl methacrylate is then run off and allowed to stand for 24 hours with slaked calcium chloride for its complete dehydration. After filtering through filter paper it is ready for thickening, but if it is not required for use immediately the monomer should be stored in a refrigerator.

Syrup Preparation.—A mixture containing 85 parts by volume of washed methyl methacrylate monomer containing 0.02% benzoyl peroxide (by weight), and 15 parts dibutyl phthalate, is placed in a flask, which should only be half filled, fitted with an air condenser. Thickening takes place in approximately 15 minutes using an oil

bath at 130° C., or in 30 minutes using a boiling water bath. Three or four shakings are necessary in each case during the process. As a precautionary measure a sink full of cold water should be at hand, so that if the reaction becomes violent during a shaking the flask can be immediately cooled. The monomer is thickened to the maximum convenient viscosity (the syrup thickens in cooling), and stored in a refrigerator until it is required for use.

Preparation of Specimens.—The fixed specimens must be thoroughly dehydrated as any residual moisture causes clouding of the "perspex." Experiments involving a final soaking in unthickened monomer before embedding, or in a 10% solution of benzoyl peroxide in chloroform, have been performed. Overnight soaking in chloroform has given best results to date. Soaking in unthickened monomer tends to retard polymerization, whereas prolonged immersion in the solution of benzoyl peroxide may cause certain gaseous reactions to take place during polymerization if this is carried out at a temperature of 37° C. or over.

Mounting.—Embedding-cells may be made from sheets of glass held together by cellulose tape. Hollow specimens and soft tissues are impregnated with monomer of slightly less viscosity than that used in embedding. A mould can be quickly and easily made by joining suitably shaped pieces of glass with "semmentum" to form a box. "Semmentum" sets quickly and will hold the glass in place in a few minutes. The mould is either left overnight or may be completely dried and hardened in a 40° C. oven in a few hours.

Embedding.—Embedding is best done in layers. First, a layer of syrup, about ¼ in. thick, is poured into the mould and polymerized at 40° C. for a few days to form a supporting layer. (Time may be saved by pouring a thinner first layer on to a sheet of ¼ in. thick "perspex" on the bottom of the mould.) Polymerization at room temperature may be carried out by ultra-violet light. Second, another layer of syrup is poured into the mould and the specimen is placed in position in it. Further layers may be needed to cover the specimen adequately if it is thick and to allow for evaporation and incomplete polymerization. A glass lid should be bound on to the mould with cellophane tape to form an air-tight cover during polymerization to prevent evaporation. When polymerization is complete the block can be shaken out of the mould or the glass broken away. The block is cut and trimmed to size and shape and finally polished by hand or on a finishing machine with the finest possible emery papers and finished with "perspex" polish (or a good metal polish).

(2) Embedding in Ward's "bio-plastic"* requires the following materials:—

Ward's "bio-plastic" ("selectron")
Tertiary butyl hydroperoxide (Ward's catalyst)

Opaque specimens are fixed and preserved in a formaldehyde fixative. Catalysed "bio-plastic" is prepared by adding 0.1%–0.5% of Ward's catalyst to the monomer. The amount of catalyst used is proportional to the size or thickness of the layer. The supporting layer is poured into the mould and allowed to gel at room temperature for one to three hours. While any bubbles are rising from the catalysed monomer, the specimen is dried by blotting with absorbent material and then air-dried, just short of shrivelling and darkening. A layer of catalysed monomer, just sufficient to cover the specimen, is then poured. The specimen is carefully placed in position so that no air bubbles are trapped. Gelling is allowed to occur at room temperature. The final layer is poured and allowed to gel at room temperature. Final polymerization is accomplished in an oven starting at 37° C. and gradually rising to, but not exceeding,

*Ward's Natural Science Establishment, Inc., Rochester 9, New York.

60° C. (The mould may be left in an incubator at 37° C. overnight.) The block is removed from the mould and shaped and polished by hand or on a finishing machine.

To get a cleared specimen it should be dehydrated with alcohol and then placed in anhydrous ether; from this it is transferred to uncatalysed monomer and the ether is slowly removed in a desiccator by slowly reducing the pressure. (It should be remembered that too rapid evacuation can cause the ether to boil and damage the specimen.) The specimen is placed into catalysed monomer and the above technique then followed.

(3) Embedding in "marco S.B.26C" resin requires the following materials:—

"Marco S.B.26C" resin
"Monomer C"
H.C.H. catalyst (1-hydroxycyclohexyl hydroperoxide-1)
"Accelerator E" (solution of cobalt naphthtnate in "monomer C")
Diethyl phthalate (plasticizer)

The plastic is prepared by mixing

A	"Marco S.B.26C" resin	100 parts	
	"Monomer C"	20 parts	
B	Catalyst	2 parts	warmed to dissolve
	"Monomer C"	20 parts	

and adding to the mixture 10 parts of plasticizer. This mixture is then filtered through glass wool. It is stable for 1 to 2 days.

"Accelerator E" (1 part) is added and the plastic is ready for polymerization.*

The remainder of the technique for opaque and clear specimens is the same as that when using Ward's "bio-plastic."

Transparent Specimens.—The following methods of mounting transparent specimens depend on the replacement of tissue fluids by fluids of a high refractive index. Transparent specimens have a certain value in demonstrating injected vessels and the bones of embryos, but are not in general very instructive. They were purchased before the second world war at high prices in Germany, but can be fairly cheaply though rather tediously prepared. Oil of wintergreen, glycerine, and paraffin have been used in their preparation. The essential features are that dehydration must be very slow and quite complete, and the longer the process the better the results.

The method which we have employed successfully is that of Spaltholz (1911) as follows. Specimens should be fixed in 10% formol saline very thoroughly, bleached in 50% H_2O_2 (but this is not essential), and dehydrated very gradually before final mounting in benzyl benzoate.

50%	Alcohol	2 weeks
60%	"	2 "
70%	"	2 "
80%	"	2 "
95%	"	2 "

The final alcohol is absolute alcohol, and two changes are made each of two weeks, the second change with a layer of anhydrous copper sulphate on the bottom of the container covered by five layers of filter paper. Two changes of benzol are required, each

*These details were kindly supplied by Mr. W. A. Norman, Wellcome Museum of Medical Science.

of two weeks, and the same procedure is followed with benzyl benzoate. The specimen is now mounted in equal parts of benzyl benzoate and oil of wintergreen. This fluid is a "perspex" solvent, and therefore glass jars must be used. Most cement seals are also dissolved, but the best seal is made of

Powdered gum arabic	50 g.
Sugar	50 g.
Sod. silicate	2 g.
Formalin	1 ml.

These ingredients are made into a thick paste with water, and after affixing the lid the jar is left undisturbed for 48 hours.

Methods using glycerine and alizarin (Fig. 12) are described by Richmond and Bennett (1938) and further reference may be made to Lee (1937) and Mall (1906).

We find that 5% gelatine with finely ground chrome yellow promotes a good injection mass for vessels in transparencies; 0.4% formalin is added just before use. Vessels should first be perfused with warm isotonic saline containing amyl nitrate to prevent vasoconstriction. Before fixation the vessels are ligated and the specimen placed in the refrigerator to set the gelatine (Fig. 13).

FIG. 12.—Transparency of a rabbit foetus prepared by Dawson's method using glycerine and alizarin. (Preparation kindly lent by Mr. J. King, London Hospital.)

Special Methods

Amyloid.—Stained amyloid tissue often fades after mounting, and to prevent fading we find the following technique valuable. The specimen is immersed in the following solution:

Iodine	1 g.
Pot. iod.	2 g.
Water	100 ml.
Sulphuric acid	1 ml.

After washing in tap water the specimen is mounted in liquid paraffin.

We have found that specimens stained with Congo Red retain their colour. After fixation in Kaiserling I specimens are immersed in 1% Congo Red for one hour and

are then transferred to a saturated solution of lithium carbonate for two minutes. They are differentiated in 80% alcohol. Normal arteries and veins tend to retain the colour. Specimens are mounted in Kaiserling III without sodium hydrosulphite

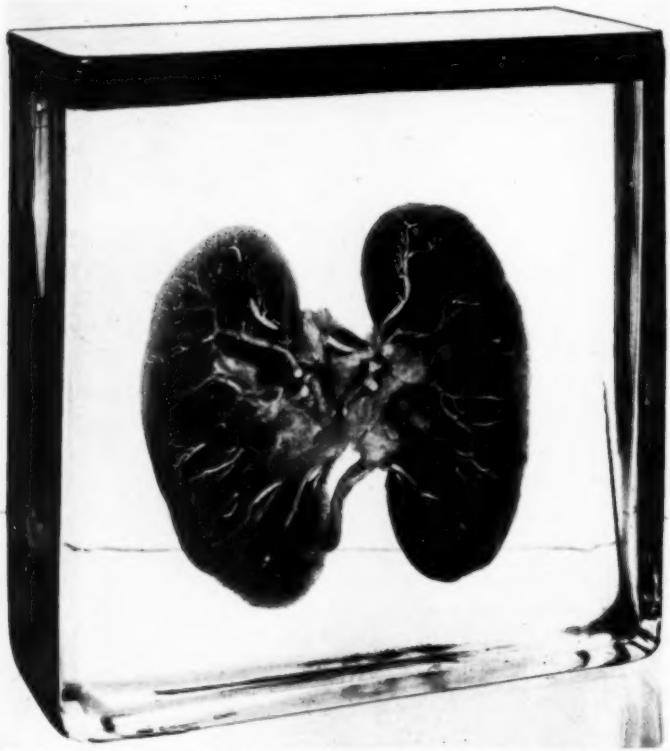


FIG. 13.—Specimen of kidney injected with chrome-yellow gelatine and cleared by Spalteholz technique to demonstrate circulatory system.

Haemosiderin.—Specimens are fixed in Kaiserling I, precautions being taken to prevent contact with iron rust. They are readily stained with a solution of equal parts of 10% HCl and 5% aqueous pot. ferrocyanide, and are then washed in running water for 12 hours. Old museum specimens of this type were mounted in 80% alcohol, as the iron diffuses in Kaiserling III. The alcohol is dangerous if specimens are opened by using a bunsen flame; and all museum curators should remember that alcohol was once a popular mounting medium.

Freshly prepared specimens should be mounted in 5% formol saline, which prevents colour diffusion except with specimens of haemochromatosis, where for some reason the colour diffuses and the fluid becomes milky within three months.

Fat may be demonstrated by staining Sudan III, and masses of malignant or other tissue by Ehrlich's haematoxylin, often an excellent way of demonstrating the limits of lesions.

Labelling of Museum Jars

In museums where specimens are used for teaching and examination it is undesirable that they should be labelled with a diagnosis. However, since a student is faced with thousands of specimens he should be enabled rapidly to determine which specimens are typical examples of common disorders, which are duplicates, and which are rare, unusual, or historical specimens.

Our practice is to label each jar with a rectangle of cellulose paint in the centre at the bottom, the label being red for common disorders, white for duplicates of common disorders, and yellow for rarities. Thus a student may cover the salient features of disease by a study of jars with red labels. The number of the specimen, preceded by a letter indicating the section of the museum to which the specimen belongs, is painted in black (Figs. 7, 8, and 9).

If "perspex" jars are used labels may be made by cutting rectangles of coloured "perspex" $\frac{1}{16}$ in. thick, and the numbers are stencilled on with "uno" stencils. "Perspex" cement is used to cover the number, and the rectangle is sealed in position with cement. If it is desired to label a specimen with its diagnosis, a "perspex" strip may be thus stencilled, covered with cement, and cemented to the centre-plate.

Catalogues

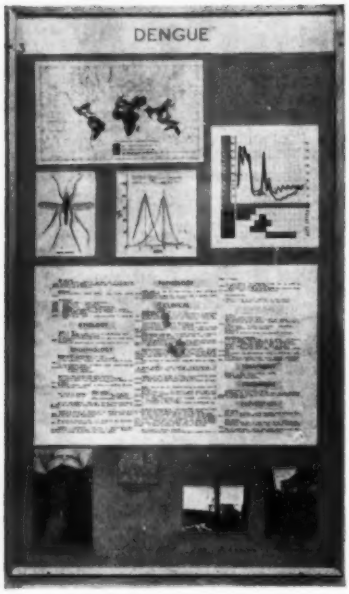
It is essential that a plan of the museum should be visible to the visitor on entering; each section should be clearly labelled. Some curators differentiate conditions in each section by a distinguishing coloured band, e.g., red for malignancy, and yellow for inflammatory processes. Two different methods of cataloguing are given by Boyd (1934) and Mundell (1935). Our own preference is for the "shannon" catalogue which holds 96 cards on wires. The edges of the cards, and thus the diagnosis of the specimen, are all simultaneously visible. Cards are easily re-arranged. We have found this catalogue to stand up to daily use for ten years. On the descriptive card a reference to the patient's case or post-mortem number is added, and in certain cases to photomicrographs of the tissue. Several duplicates of each catalogue should be available.

Lighting

A poorly lighted museum is useless, and in any museum the best lighted exhibits are the ones most frequently examined. Fluorescent lighting (Karsner, 1941) is the best available, giving at least fifty per cent better results than were previously available.

Methods of Presentation

We are still far from the ideal museum of pathology. In every museum there are specimens of great value, mounted with technical precision, and fully apt to illustrate a lecture or inform a visitor. But the ideal pathological museum should present a student with the full picture of human disease; it should show, by photographs, the appearance of the patient, and the aetiological and environmental factors that lead to disease. Some attempt should also be made to impress upon students the statistical significance of each disorder, and the changes which



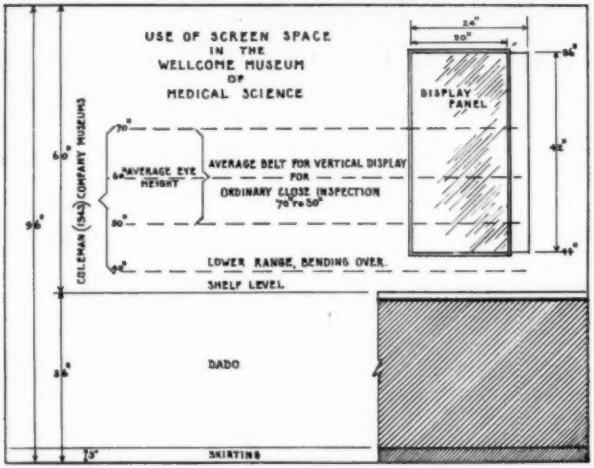


FIG. 14.—Series of photographs illustrating the method of presentation of material at the Wellcome Museum of Medical Science, London. (Photographs kindly lent by Dr. C. J. Hackett.)

are taking place in the incidence of each. *X*-ray and laboratory findings should be illustrated. Not least there should be photographs of the men and the women who have, by their labours, established clinical entities and evolved methods of treatment and diagnosis. Somewhere also there should be room for a few sentences from the writings of those who were inspired to make original and fundamental observations. Perhaps if each museum exhibited, as some few do, a memorable saying of a great writer publicly displayed as a motto, a step might be taken to make museums more important in the education of students. In the writings of Sir Thomas Browne and of Montaigne are to be found innumerable sentences which seem to define the spirit of medicine.

In Great Britain the Wellcome Museum of Medical Science, under the direction of Daukes (1929, 1936), has set a standard which few have approached and none excelled. It is not possible to give full references to American trends, but a discussion of these is given by Aegerter (1948).

In the Wellcome Museum the basic principle is that the belt of vision lies between 40 in. and 70 in. from the ground. There is a colour code throughout for aetiology, epidemiology, pathology, clinical treatment, prognosis, and prevention. Displays, in the form of a very large book with hinged frames, are available and frequently changed (Fig. 14). Methods of display are discussed by Greenwood (1944).

The mounting of lung specimens on transparent sheets introduced by Gough (1947) has inspired Benians (1948) and his colleagues to develop a miniature museum in portfolios. After fixation, specimens are cut by Anderson's method and mounted in gelatine in " perspex " frames. Folios are preserved in covers with histories and all the relevant clinical data, references for further reading, and photomicrographs and tracings of the specimen with explanatory notes. This method offers an enormous saving of space, and although there is some sacrifice of reality in losing the three dimensional presentation of a mounted specimen, hollow viscera not being suitable for this technique, there is little doubt that most museums will in future present such exhibits as ancillaries. We have found that stained giant sections mounted in the ordinary way in Canada balsam and other histological media are very valuable, and are more easily prepared than is generally recognized.

The scope of the medical illustration has been discussed by Treadgold (1949), where the practice of Guy's Hospital Medical School is given. The use of cartoons, and what was called by a later correspondent " education by terror," are innovations not likely to commend themselves to traditionalists, but they have long been in use in Vienna and in the German schools. Much attention is rightly given to the type of script, the use of colour, and the making of models. Moulage has in fact a great value in museums, which cannot for obvious reasons present specimens of hands or face. Material for this purpose was previously readily available in Germany, and we have not found the technique laborious; there is a need for its reintroduction in this country. The older museums present admirable models in wax. In certain American hospitals, e.g., Rochester General Hospital, specimens have been replaced by " kodachrome " transparencies, a practice not likely to be widely followed.

Some compromise between a collection of specimens for teaching purposes and a display intended to present the picture of disease as a whole is possible by the selection of certain disorders for special attention. For example, at Westminster Medical School we have built display cabinets in tiers with illuminated panels. In these a condition such as syphilis is illustrated, its history briefly shown, the statistics of its incidence graphically outlined, photographs of patients and specimens of lesions given, and letters from patients suffering from G.P.I. are exhibited (Fig. 15). Similar exhibits are offered for tuberculosis and malignant

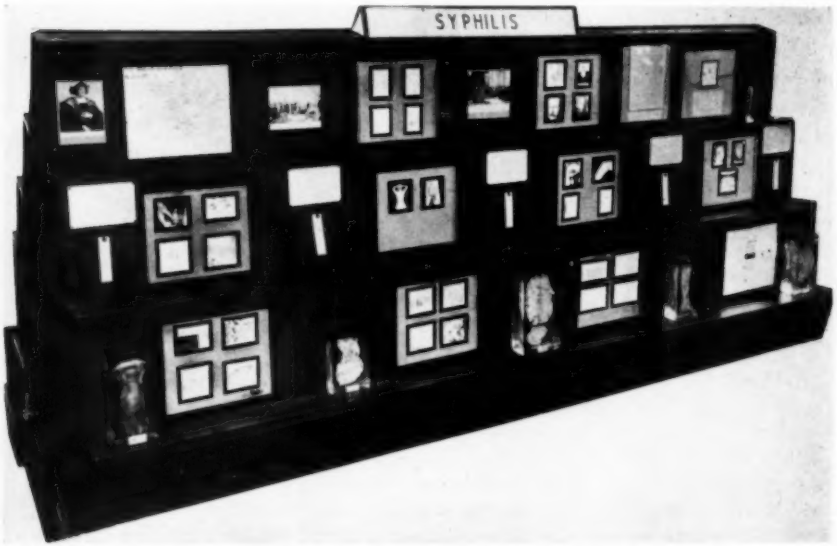


FIG. 15.—Central display cabinet in Westminster Medical School museum showing semi-permanent display on syphilis.

disease. Exhibits of this kind make great demands on the imagination and the time of curators. Much may be learned from commercial exhibitors. The art of advertising (Symes, 1948) has much to teach the medical teacher. A department of medical photography is essential, and its director must be prepared to study the technique of instantaneous impressiveness. Vivid colours, geometrical forms, epigrammatic script may seem childish devices, but material exhibited with their help may summarize once and for all a basic principle.

Finally, who should direct the policy of a museum and determine its content ? It is usually the function of a pathologist, who must in any case have the last word. But a pathologist is often more interested in curiosities than in the every-day conditions, and in any case is only one of the teachers who use the material. His colleagues only too often wish him to include specimens whose removal was a personal triumph, or may believe that even a hundred specimens of their own

favourite lesion are not excessive. Consequently it is essential that there should be for each section of the museum a clinician with particular knowledge of the system studied, and the advice of clinicians should always be sought. Financial support for museums of pathology in Great Britain has been liberal, and only the initiative of curators and the limitations of space may impair their quality.

References

Aegerter, E. E. (1941). *Amer. J. clin. Path.*, **11**, Tech. Sect., 147.
—— (1948). *Bull. int. Ass. med. Mus.*, **28**, 167.
Anderson, J. (1929). *How to Stain the Nervous System.* Edinburgh.
Baker, S. L. (1940). *J. tech. Meth.*, **20**, 42.
Bartoe, W. F. (1940). Ibid., **20**, 6.
Benians, T. H. C. (1948). *Lancet*, **2**, 983.
Boyd, W. (1934). *J. tech. Meth.*, **13**, 27.
Brown, A. F., and Schroeder, M. F. (1937). Ibid., **17**, 59.
Carroll, P. L., and Neidhoefer, J. R. (1936). *Science*, **83**, 289.
Culling, C. (1949). *Bull. med. Lab. Tech.*, **14**, 93.
Daukes, S. H. (1929). *The Medical Museum.* London.
—— (1936). *J. tech. Meth.*, **15**, 9.
Davis, C. L. (1942). Ibid., **22**, 57.
Duff, G. L., and More, R. H. (1944). Ibid., **24**, 1.
Dukes, C., and Bussey, H. J. R. (1936). Ibid., **15**, 44.
—— and McDonnell, R. J. C. (1938). Ibid., **18**, 62.
Gough, J., and Wentworth, J. E. (1949). *J. R. micro. Soc.*, **69**, 231.
Greenwood, H. W. (1944). *B. J. Photography*, p. 318.
Hall, W. E. B. (1944). *J. tech. Meth.*, **24**, 12.
Hibben, J. H. (1937). *Science*, **86**, 247.
Hill, W. C. O. (1937). *Ceylon J. Sci.*, **4**, 147.
Jores, — (1913). *Ver. dtsch. Path. Gesellsch.*, **16**, 357.
Kaiserling, C. (1897). *Virchows Arch.*, **389**, 147.
Karsner, H. T. (1941). *J. tech. Meth.*, **21**, 26.
Klotz, O., and Maclachlan, W. W. G. (1915). Ibid., **5**, 59.
Kramer, F. M. (1940). Ibid., **20**, 14.
Larson, C. P., and Levin, E. J. (1940). *Arch. Path.*, **30**, 1093.
Lee, A. B. (1937). *The Microtomist's Vade-Mecum.* London.
Leibow, A. A., Hales, M. R., Lindskog, G. E., and Bloomer, W. E. (1947). *Bull. int. Ass. med. Mus.*, **27**, 116.
Lewis, H. D., and Gaines, W. E. (1936). *Anat. Rec.*, **65**, 377.
Lieb, E. (1940). *J. tech. Meth.*, **20**, 48.
Lundy, J. S., and Camp, J. D. (1939). Ibid., **19**, 58.
Mall, F. P. (1906). *Amer. J. Anat.*, **5**, 433.
Mundell, C. D. T. (1935). *J. tech. Meth.*, **14**, 52.
Narat, J. K., Loef, J. A., and Narat, M. (1936). *Anat. Rec.*, **64**, 155.
Pulvertaft, R. J. V. (1936). *J. tech. Meth.*, **16**, 27.
Richmond, G. W., and Bennett, L. L. (1938). *Stain. Tech.*, **13**, 77.
Robertson, H. E., and Lundquist, R. (1934). *J. tech. Meth.*, **13**, 33.
Sahasrabudhe, N. S. (1934). Ibid., **13**, 43.
Schultz, A. (1931). *Klin. Wschr.*, **10**, 213.
Smith, J. R., and Henry, M. J. (1945). *J. Lab. clin. Med.*, **30**, 462.
Spaltholz, K. W. (1911). *Uber das Durchsichtigmachen von menschlichen und therischen Präparaten.* Leipzig.
Strumia, M. M., and Hershey, J. I. (1948). *J. Lab. clin. Med.*, **33**, 1311.
Symes, H. (1948). *Selling through Display.* H.M.S.O.
Treadgold, S. (1949). *Lancet*, **256**, 701.
Vowles, A. H. (1942). *J. tech. Meth.*, **22**, 61.
Wagoner, G., and Nuckols, H. H. (1935). Ibid., **14**, 35.
Wentworth, J. E. (1942). *J. Path. Bact.*, **54**, 137.
—— (1947). *Bull. int. Ass. med. Mus.*, **27**, 201.

J. clin. Path. (1950), 3, 24.

THE RED BONE MARROW IN HEART FAILURE

BY

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It is generally accepted that the normal amount of red marrow in the femur of healthy male adults does not exceed a few flecks or patches in the upper end of the bone. In the female this appearance is not so consistent, and the haemopoietic marrow may occupy up to a half or two-thirds of the shaft without evidence of anaemia or excessive blood loss from menstruation, haemorrhoids, or other explanatory cause (Turnbull, 1934).

In the routine examination at necropsy of the femora of all patients, both male and female, occasional examples display a great increase in the amount of red marrow, unassociated with anaemia or other obvious cause. On further study it appeared that this increase was closely associated with certain types of heart failure. This paper describes the results of analysis of the naked eye description of the femoral bone marrow at necropsy of 200 selected subjects, including various types of heart failure, and normal controls.

Some of the specimens were examined personally ; for the rest it was necessary to consult the records of the Institute. In order to obviate discrepancies that might arise from variation in the observations of different reporters it was decided to use the reports of one pathologist (Dr. W. W. Woods) only.

For this analysis it was felt desirable first to separate males from females, and to subdivide these into smaller groups, representing the various types of heart disease associated with cardiac failure. Where possible, 20 cases of each group were studied (Table I).

TABLE I

	No. of Cases	
	Males	Females
Controls	20	20
Hypertension without cardiac failure	20	20
Hypertension with cardiac failure	20	20
Mitral stenosis with cardiac failure	20	20
Emphysema with cardiac failure	20	11
Aortic valve disease with cardiac failure	16	0

Controls

The records of the amount of red marrow found in the femora of 20 male and female patients were examined. The subjects were all over the age of 40, the oldest being 73. Death was due either to accidental trauma or to surgical operation, and careful note was made of the clinical and pathological records to exclude any disease that might be responsible for a variation in the amount of haemopoietic marrow. Of the males, one had confluent red marrow in the upper part of the shaft, head, and neck, 13 showed a few flecks or patches in the upper part of the bone, while six were fatty throughout (Figs. 1 and 3). The female group, on the other hand, demonstrated the erratic increase of haemopoietic tissue described by Turnbull (1934); thus 12 showed confluent red marrow in the upper part of the shaft, head, and neck of the bone, and the rest a few flecks and patches (Fig. 9).

FIG. 1.—Right femur from a control male subject aged 61, showing a few flecks and patches of red marrow in the upper part of the shaft, head, and neck.

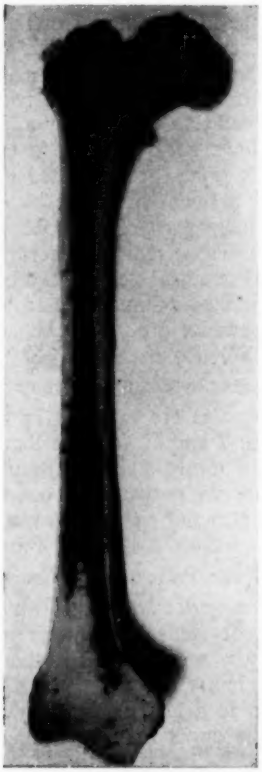


FIG. 2.—Right femur from a female subject, aged 63, who died from hypertensive heart failure, showing great increase of red marrow.

Hypertensive Group

In subjects with essential hypertension, either benign or malignant, dying from causes other than heart failure (usually either cerebral haemorrhage or intercurrent acute illness), the amount of red haemopoietic marrow in the femora showed little variation from that in the control groups. The subjects studied were all over the age of 40. The males showed a few flecks or patches in the head, neck, and upper part of the shaft (Fig. 4), and of the female femora three had areas of confluent red marrow and the rest flecks or patches in the greater part of the shaft, head, and neck (Fig. 10).

Where heart failure had been associated with the hypertension for periods

of at least one month before death, both males and females showed a marked increase in the haemopoietic marrow. The criteria accepted for such heart failure were primarily clinical, and consisted of severe dyspnoea on exertion, or at rest, with corresponding physical signs of heart failure; confirmation was provided by the organs at necropsy. Eighteen of the males showed confluent red marrow in the head, neck, and up to two-thirds of the length of the shaft of the femur (Fig. 5). The females showed a corresponding increase with confluent red marrow through the greater part of the bone in all cases except one which had many patches in the upper two-thirds (Fig. 11).

Mitral Stenotic Group

The examination of the bone marrow of this group showed that, although there was a significant increase in the amount of the haemopoietic tissue, it was not as great as in the hypertensive subjects (Figs. 6 and 12). Only six of the 20 male patients examined showed confluent red marrow in the head, neck, and shaft of the bone, 12 had flecks or patches, and two were fatty throughout. In some of these cases it was noted that the marrow appeared oedematous on naked eye examination; this may have been a factor in suppressing the haemopoietic response in these. The female group demonstrated a parallel increase in the red marrow which again was not as great as in the hypertensive cases. Fifteen had confluent red marrow in the upper part of the bone, and the other five a number of flecks and patches. The absence from our necropsy material of a statistically significant number of cases where mitral stenosis had been unaccompanied clinically by evidence of heart failure makes it impossible to present a parallel series such as was provided in the hypertensive group.

Emphysematous Group

Twenty males and 12 females are included in this series, and all exhibited cardiac failure clinically. Figs. 7 and 13 indicate that the increase of red marrow closely approaches that in the mitral stenotic group. Of the males, six showed confluent red marrow in the upper part of the bone with flecks or patches in the rest, and of the 12 females examined eight had red marrow in the upper part of the bone.

Aortic Valve Disease Group

Sixteen male subjects who had died from heart failure secondary to a pure aortic valve lesion, either aortic stenosis or aortic incompetence, are charted in Fig. 8. The aetiological factors responsible for these lesions include rheumatic heart disease, syphilis, and primary atheroma. In eight there was an increase in the red marrow, involving up to a half or two-thirds of the shaft. The rest had a few patches or flecks in the upper end. A comparable group of females was not obtained.

Microscopical Histology

In two of the cases of hypertensive heart failure showing this hyperplastic marrow macroscopically, the microscopic examination has yielded no information beyond an increased normoblastic proliferation.

Discussion

A search of the literature has revealed no reference to naked eye observations on the red marrow in the various types of cardiac failure.

Weil (1901) drew attention to the polycythaemia of the peripheral blood in two children suffering from congenital heart disease, and noted that the marrow appeared very red and cellular.

Mackay (1907) confirmed these observations in an adult dying of congenital pulmonary stenosis. In antithesis he noted that in a middle-aged man dying from pulmonary emphysema, who was cyanosed in life without polycythaemia, the bone marrow at necropsy appeared normal. Parkes Weber (1913) found a decided erythroblastic reaction in the bone marrow of a woman aged 29 who, in life, had suffered from emphysema with cyanosis ; at necropsy the greater part of the bone marrow of the humerus was of a dull, earthy-red colour.

Several observers have described polycythaemia without splenomegaly in association with hypertension. Geisböck (1904), in examining the blood of patients with increased blood pressure, found that the number of red cells was greatly increased, and his observations have been confirmed by Hess (1905), while Howard (1908) described one case of hypertension in association with polycythaemia but with no splenomegaly. Parkes Weber (1921) in a review of this condition of " polycythaemia hypertonica " quotes several cases, and considers it not uncommon. He believes that the polycythaemia is almost certainly secondary.

None of these observers has considered the influence of cardiac failure on the amount of polycythaemia, and, if our present observations on the amount of red marrow reflect what is happening in the peripheral blood, hypertension alone does not cause red marrow hyperplasia, but the superimposed heart failure is responsible. Groen and Godfried (1948) reported a series of cases of severe heart failure in which there was an increase in the number of circulating normoblasts in the blood, and concluded that this was a grave prognostic sign.

It is generally assumed that the stimulus to the haemopoietic system is low oxygen tension (Wintrobe, 1946) ; but in our series of cases the greatest hyperplasia was observed in the marrows of the hypertensive group of patients, who, on the whole, are less cyanosed and have a higher oxygen tension than the emphysematous or mitral stenotic groups. It seems likely that some factor other than anoxia is responsible for this variation. Dacie and White (1949) suggest that the actual flow of blood through the marrow is a factor which possibly affects erythropoiesis by controlling the delivery of erythrocytes into the circulation from the sinusoids, and perhaps even affects erythropoietic activity itself. Grant and Root (1947), working with dogs, observed an increased erythropoiesis that lasted for three weeks following loss of 30% of the blood volume, but the lowered oxygen tension and saturation of the bone marrow lasted from three to five hours only. Howarth and McMichael (1946) showed that hypertensive cardiac failure may be accompanied by increased peripheral resistance, which is diminished when the failure is relieved. Davis (1941) produced polycythaemia in rabbits by the administration of ephedrine ; it therefore seems possible that local vascular spasm may play a part in the increase in the haemopoietic marrow.

Summary

Observations on the amount of red marrow found in the femur at necropsy in various types of heart disease have been recorded.

Essential hypertension alone has no effect on the haemopoietic marrow, but when cardiac failure has been present there is a marked increase in the red marrow.

This increase of red marrow, although present, does not occur to such an extent in heart failure associated with mitral stenosis or emphysema.

I wish to thank Professor Dorothy Russell for suggesting this investigation and for her help and encouragement in following it up; Dr. W. W. Woods for his observations on necropsies; and Mr. John King for the photographs.

REFERENCES

Dacie, J. V., and White, J. C. (1949). *J. clin. Path.*, **2**, 1.
Davis, J. E. (1941). *Amer. J. Physiol.*, **134**, 219.
Geisböck, F. (1904). *Ver. Kongr. f. Inn. Med. Wiesbaden*, **21**, 97.
Grant, W. C., and Root, W. S. (1947). *Amer. J. Physiol.*, **150**, 618.
Groen, J., and Godfried, E. G. (1948). *Blood*, **3**, 1445.
Hess, O. (1905). *Med. Klinik, Vienna*, **1**, 68.
Howard, T. (1908). *J. Amer. med. Ass.*, **51**, 1230.
Howarth, S., McMichael, J., and Sharpey-Schafer, E. P. (1946). *Clin. Sci.*, **6**, 41.
Mackey, L. G. J. (1907). *Bgham. med. Rev.*, **10**, 113.
Turnbull, H. M. (1934). *The Anaemias*. Ed. Vaughan, J. M. Oxford University Press. London. P. 11.
Weber, F. P. (1913). *Proc. R. Soc. Med.*, **6** (Sect. Med.), 83.
—— (1921). *Polycythaemia, Erythrocytosis, and Erythraemia*. Lewis. London.
Weil, E. (1901). *C. R. Soc. Biol., Paris*, **53**, 713.
Wintrobe, M. M. (1946). *Clinical Haematology*. 2nd ed. London. Henry Kimpton.

MALES
CONTROLS

CONTROLS

MALES

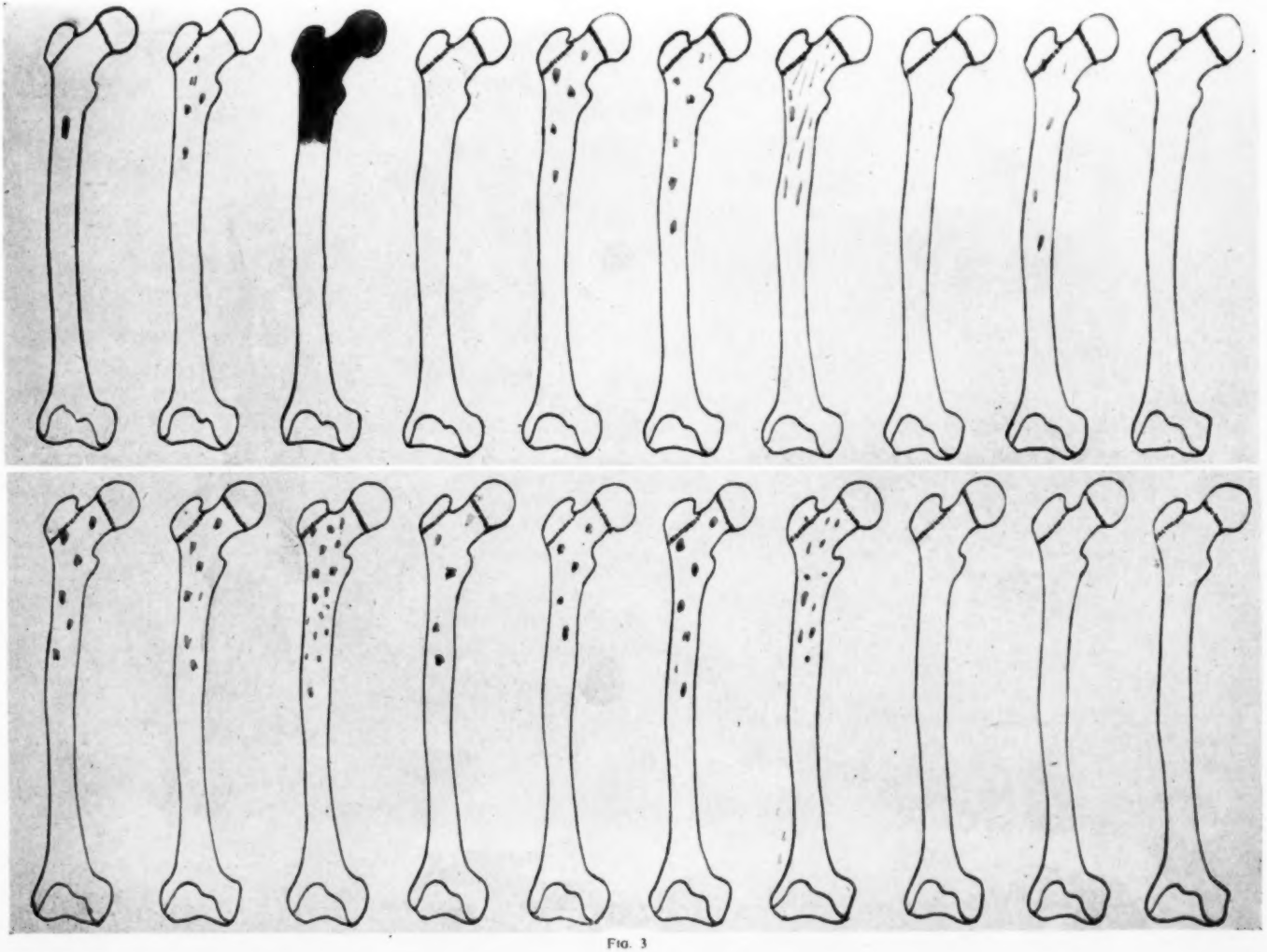


FIG. 3

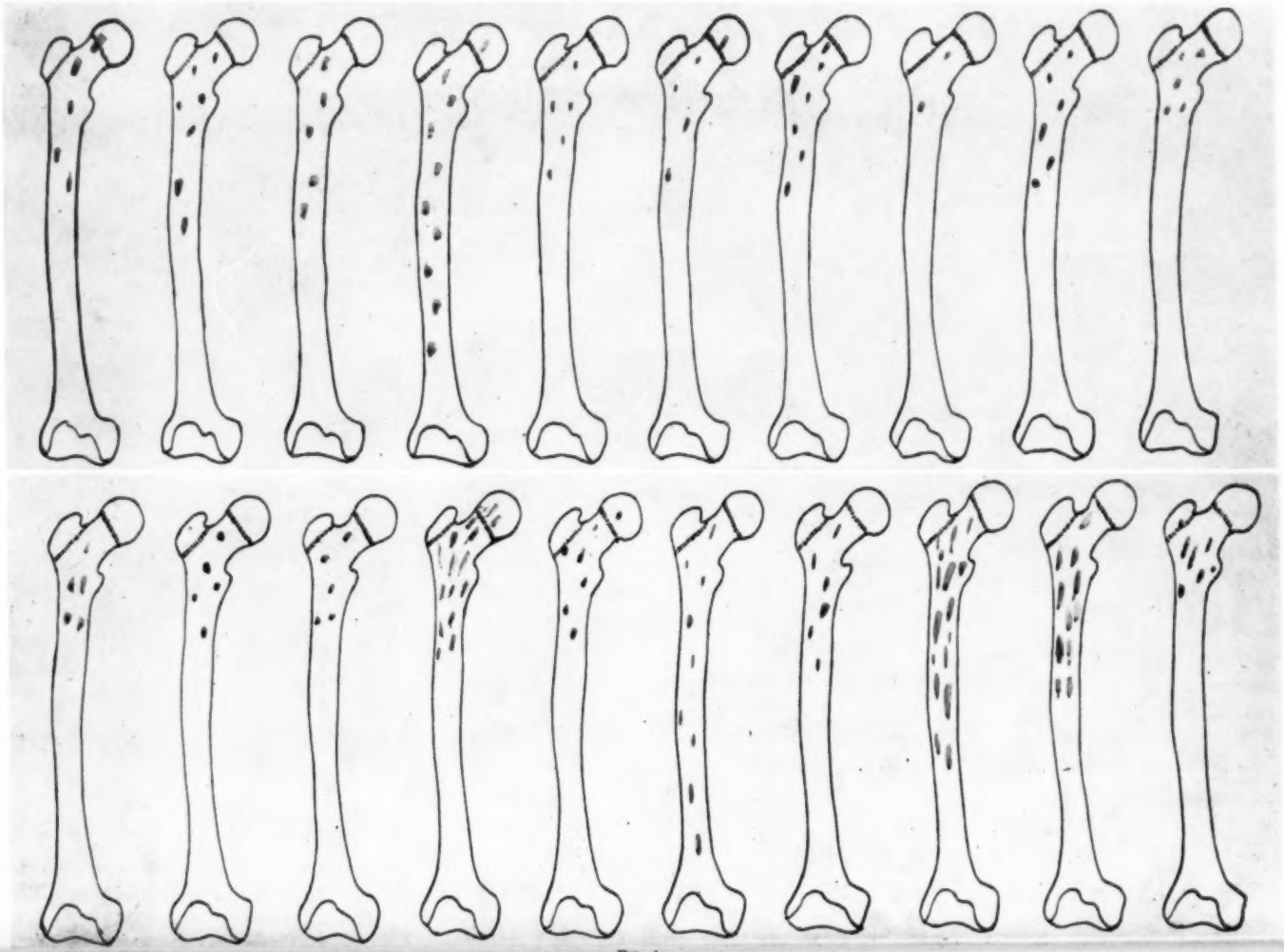


FIG. 4

FIG. 4

HYPERTENSION WITH CARDIAC FAILURE MALES

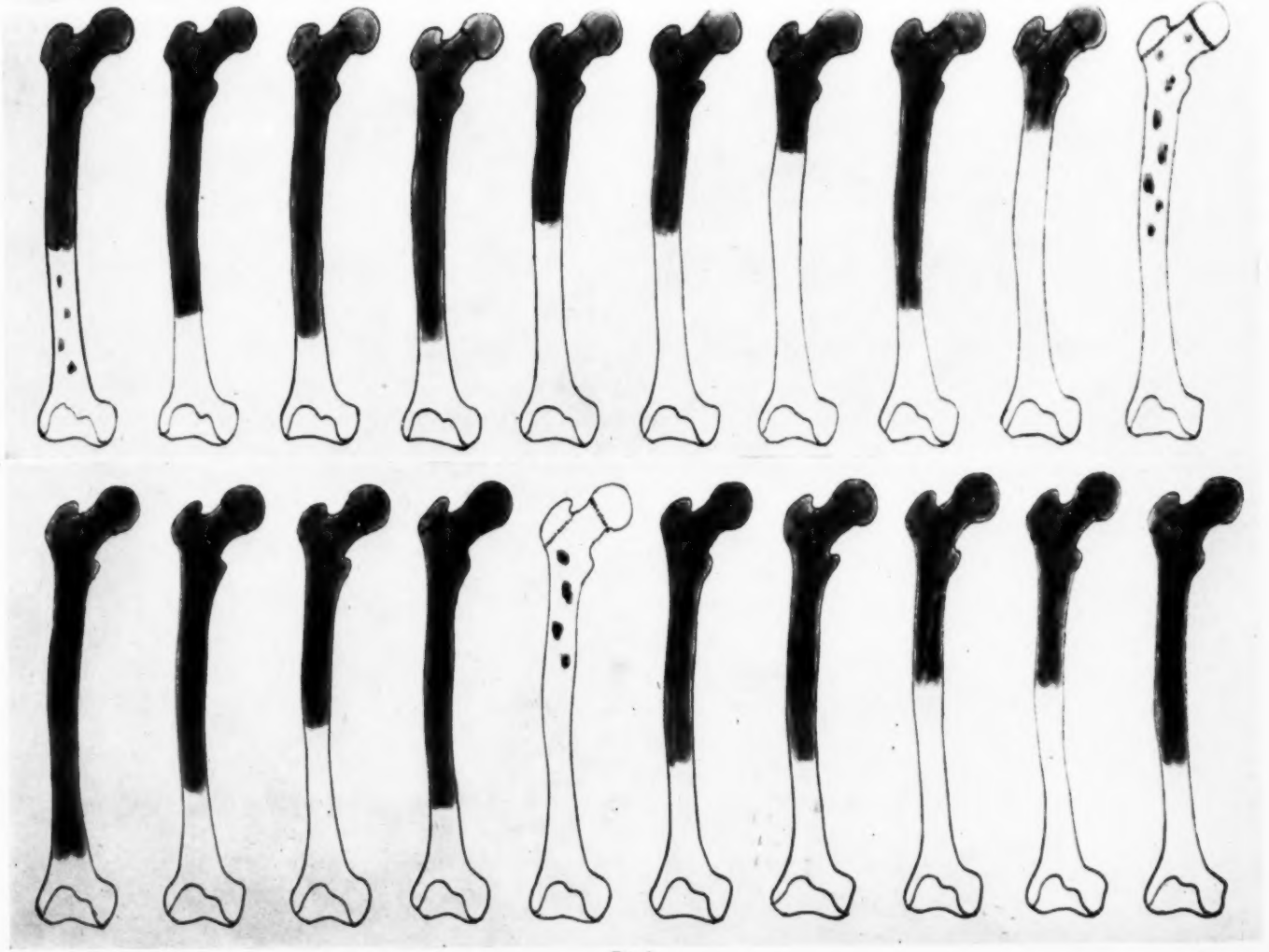
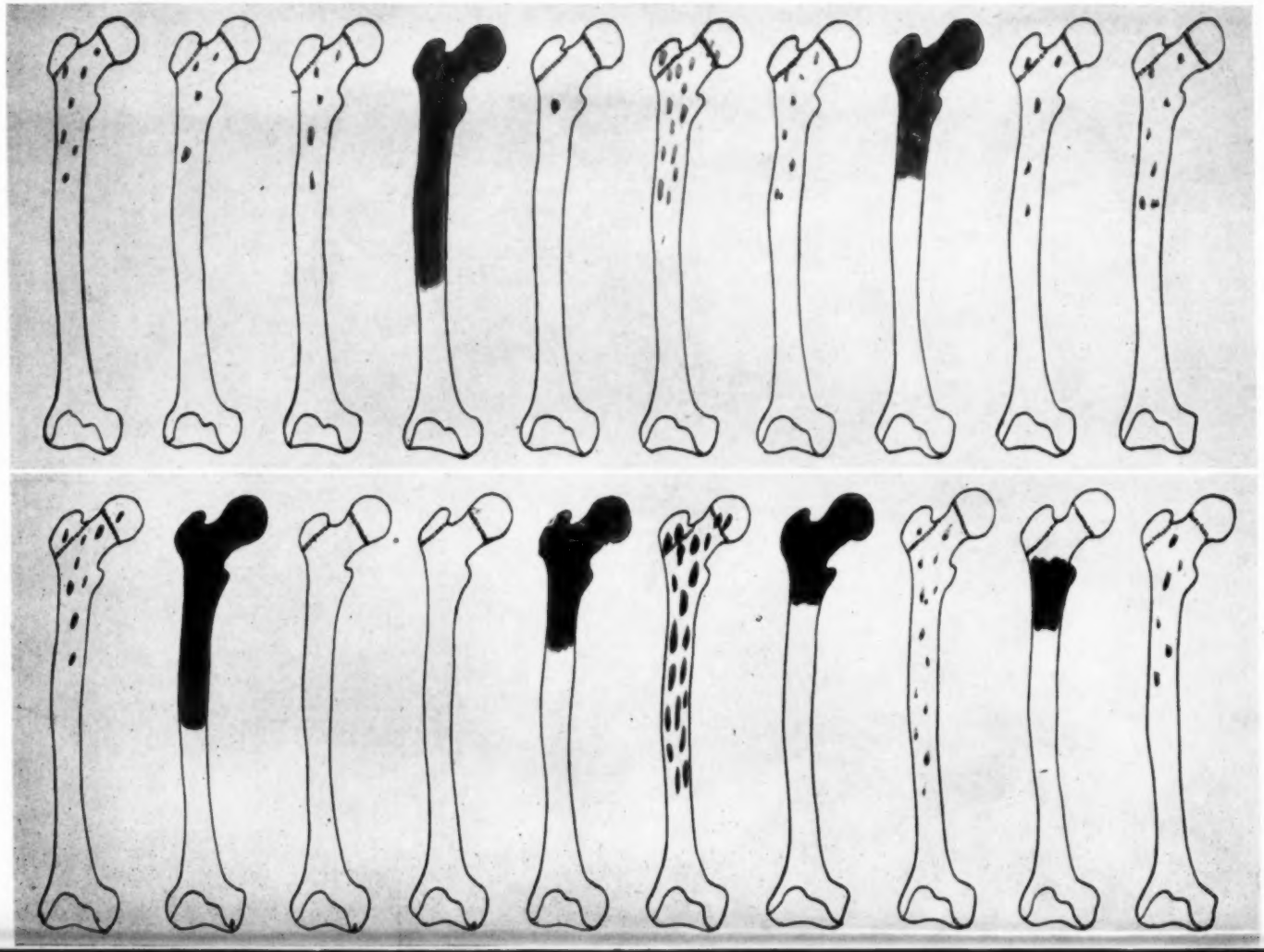


FIG. 5



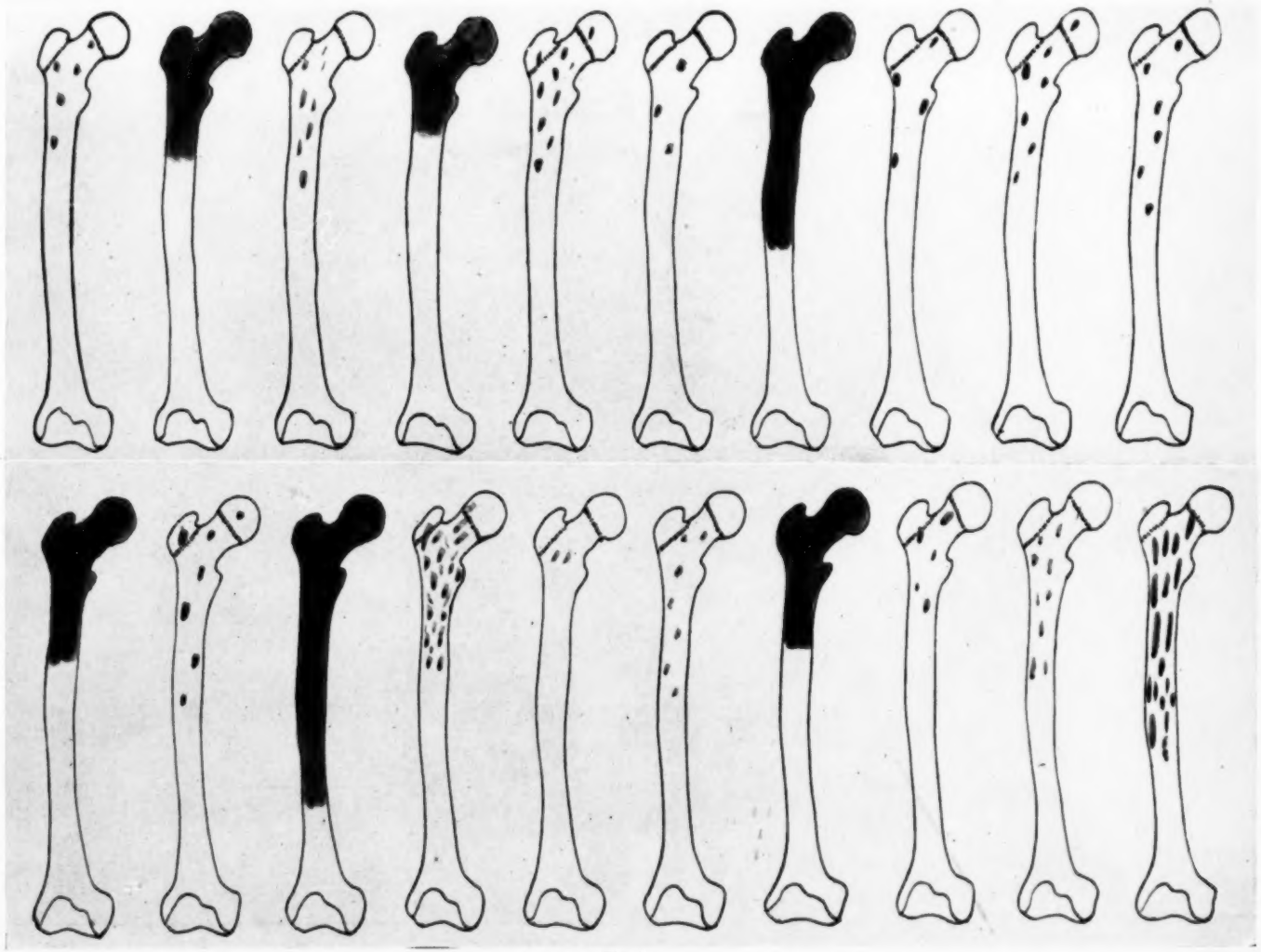
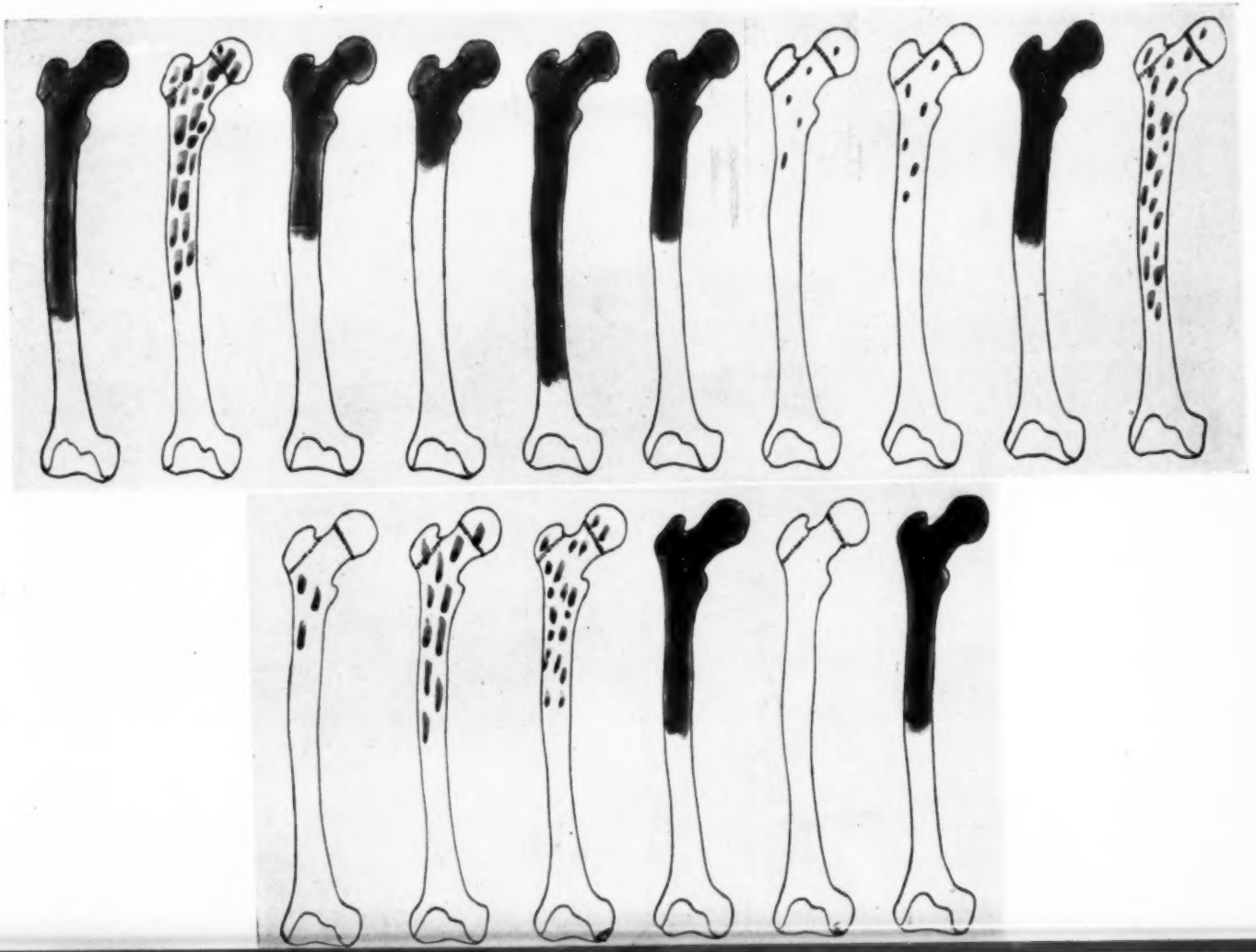


FIG. 7

AORTIC VALVE DISEASE WITH CARDIAC FAILURE

MALES



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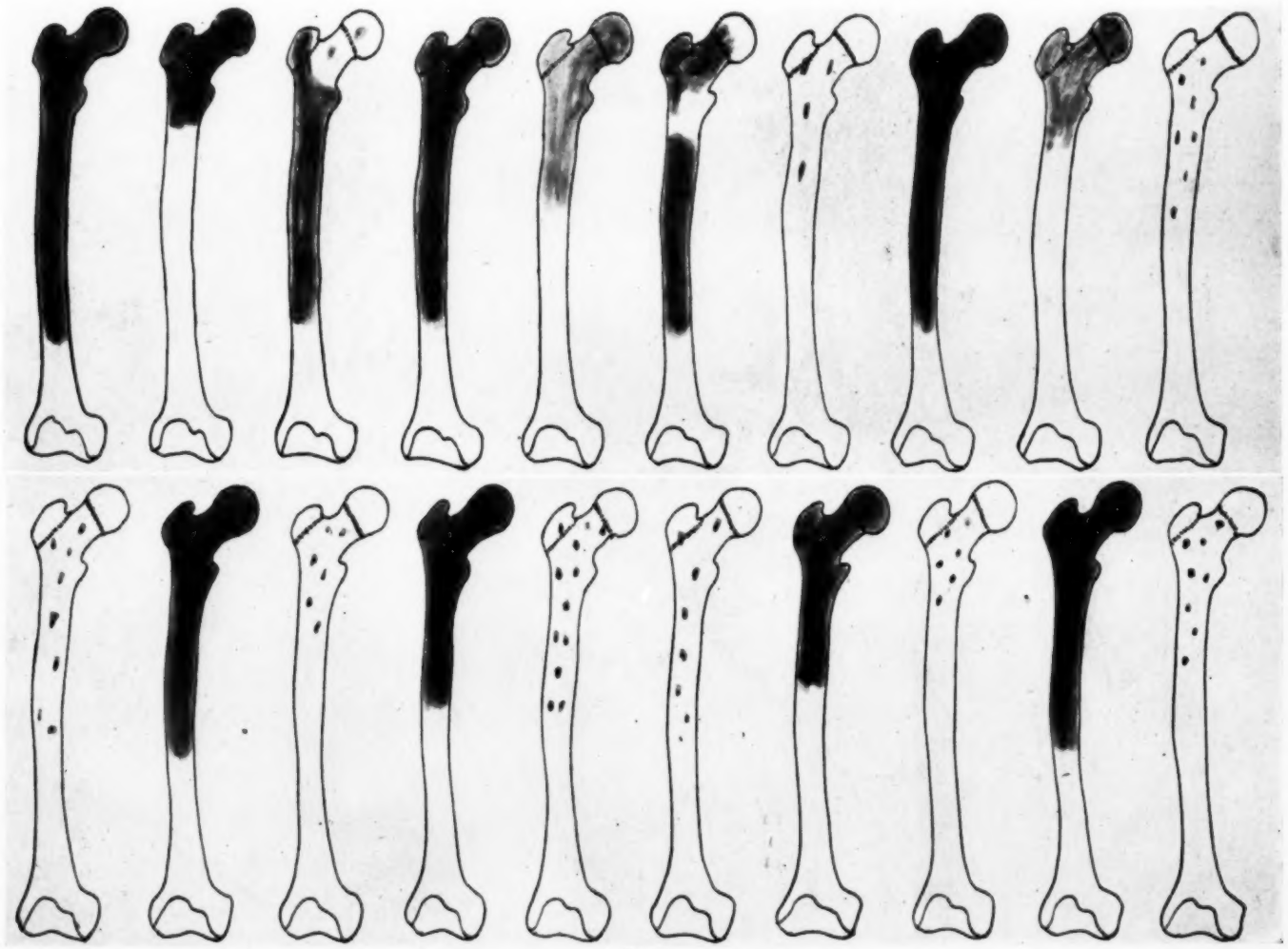


FIG. 9

HYPERTENSION WITHOUT CARDIAC FAILURE

FEMALES

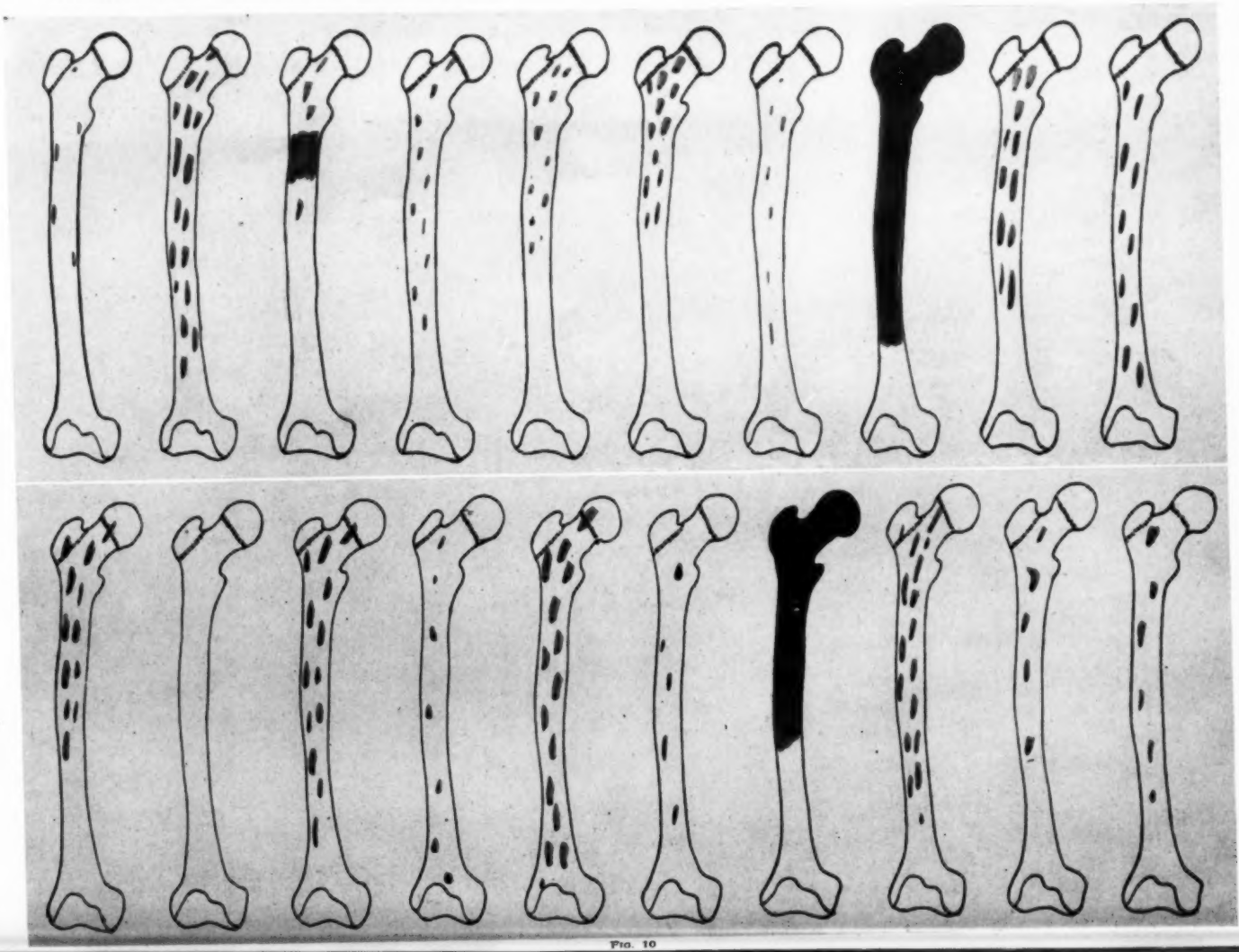


FIG. 10

HYPERTENSION WITH CARDIAC FAILURE

FEMALES

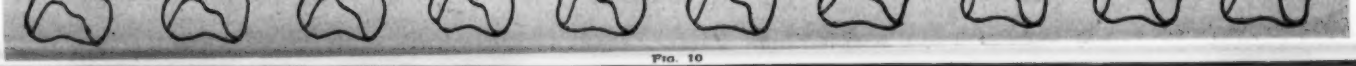


FIG. 10

HYPERTENSION WITH CARDIAC FAILURE

FEMALES

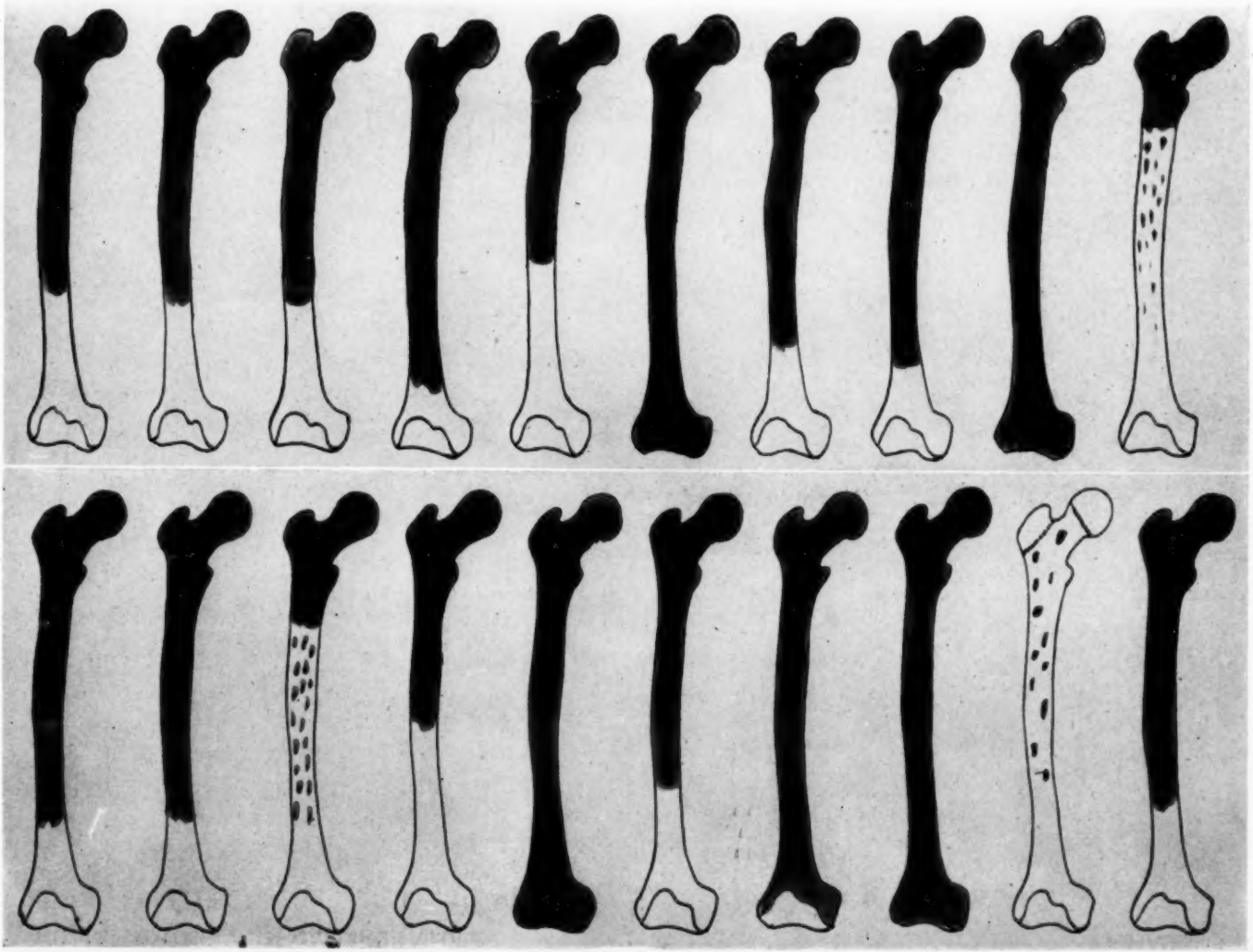


FIG. 11

MITRAL STENOSIS WITH CARDIAC FAILURE

FEMALES

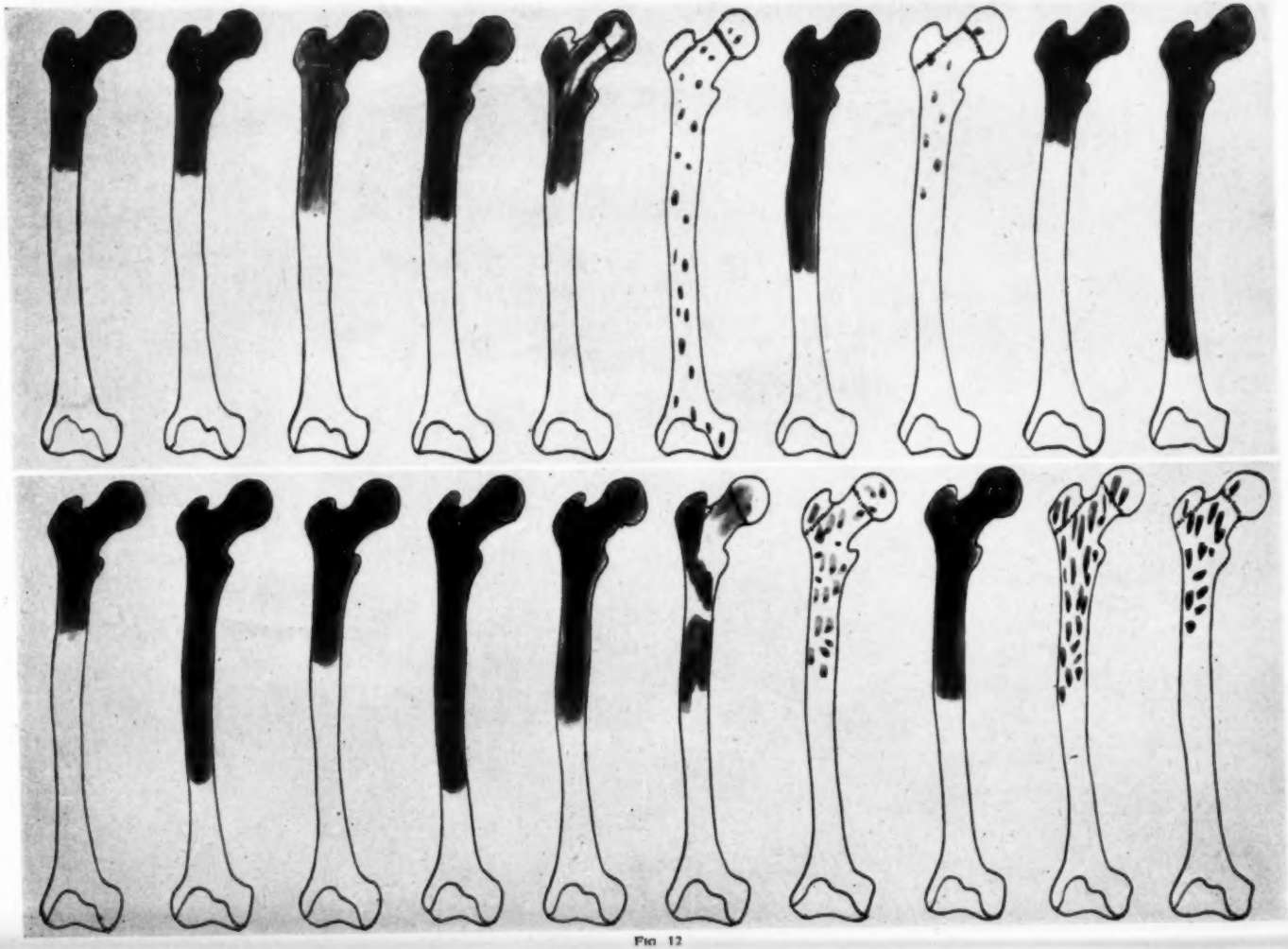


FIG 12

MITRAL STENOSIS WITH CARDIAC FAILURE

FEMALES

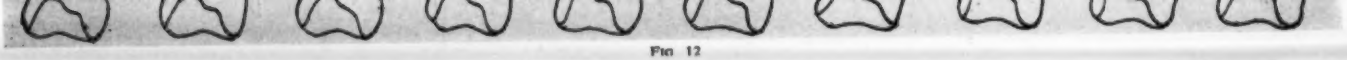


FIG. 12

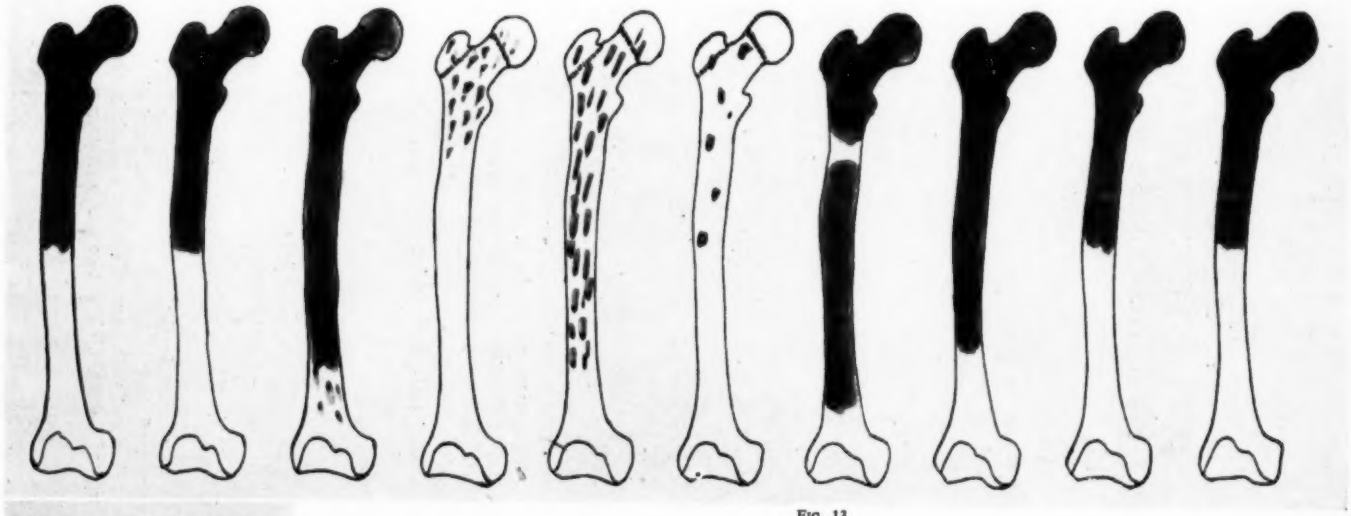
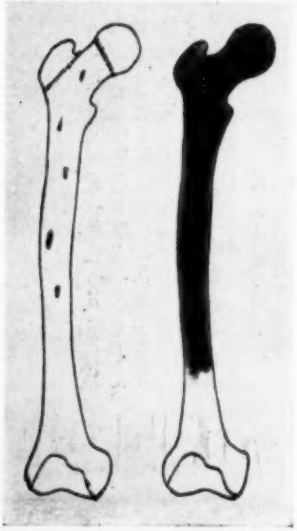


FIG. 13



J. clin. Path. (1950), 3, 40.

THE DIAGNOSIS OF NEOPLASTIC CELLS IN SPUTUM BY TWO NEW METHODS

BY

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The following stains have been used in other laboratories for the demonstration of neoplastic cells in sputum: (1) haematoxylin and eosin (numerous workers including Dudgeon and Wrigley, 1935); (2) haematoxylin, orange G, light green, eosin, bismarck brown (Papanicolaou, 1942); (3) methylene blue (Schuster, 1947).

The use of the first two methods has the disadvantage that the procedure is time-consuming, since the preparation has to be fixed before it is stained, and the amount of sputum which can be examined on one slide is extremely small. The last method is rapid, gives results that compare favourably with Papanicolau's method in proved cases of carcinoma, enables a larger quantity of sputum to be examined on one slide, but requires considerable experience to differentiate neoplastic from normal cells. It was thought that a counter-stain for cytoplasm might make diagnosis simpler, especially in the squamous cell types of carcinoma, which form at least 50% of the various morphological types encountered in sputum. Many counter-stains were tried, but most of them precipitated when mixed with the methylene blue, or failed to stain cytoplasm in vital preparations. Finally basic carbol fuchsin was found satisfactory in the proportions given below. Carbol fuchsin stains normal pharyngeal squamous epithelium brilliant red in bacteriological preparations, and it seemed likely that it would stain thickly keratinized carcinoma cells even more intensely.

Method

The following solutions are required:

A.	Methylene blue	0.5 g.	B.	Basic fuchsin	1.0 g.
	Glycerin	20 ml.		Absolute alcohol	10 ml.
	Distilled water	80 ml.		5% carbolic acid	90 ml.

One part of B is mixed with four parts of A. These proportions may be varied slightly for different batches of stain.

The method of preparation of films of sputum is similar to that described by Schuster (1947). An early morning specimen of sputum is emptied into a petri dish. Purulent or blood-stained fragments are picked out with forceps against a black background, and placed on a slide with a drop of the stain. The two are gently warmed

on a bunsen flame, and mixed with the blade of the forceps on the slide for at least half a minute. The uniformly stained, sticky specimen is covered with a large cover slip and spread evenly by gentle pressure. Staining improves with time, but the specimen can be examined almost immediately. (In all the vital staining methods we have tried, the staining of the preparation if left for up to six hours may become so intense that earlier nuclear detail is obscured, although diagnosis need not necessarily be more difficult.) The field is searched with a 2/3 objective, and suspected areas are examined more closely with a good sixth or seventh objective.

FIG. 1.

FIG. 2.

FIG. 1.—Spheroidal cell carcinoma. (× 800. Iodine green and neutral red.)
FIG. 2.—Partly oat, partly spheroidal cell carcinoma. (× 800. Iodine green and neutral red.)

The carbol fuchsin methylene blue stain has proved the stain of choice in the diagnosis of the group of squamous cell carcinomata, but, because the nuclear detail in oat and spheroidal cell types of carcinoma was not as good as in fixed films stained by more usual methods, a search was made for a suitable alternative stain to supplement the methylene blue fuchsin. A 2% solution of watery iodine green* was found eminently satisfactory, and proved a great advance on either methylene blue alone or combined with carbol fuchsin. The technique of preparation of the specimen is similar to that already described. A drop of iodine green is mixed with the sputum on the warmed slide. If one part of 2% iodine green is added to 20 parts of 1% watery neutral red, the nuclear detail is further enhanced, the chromatin appearing as a crisp, black lace-work pattern, the cytoplasm remaining green (Figs. 1 and 2). This mixed stain has one disadvantage in that it will precipitate in the preparation in two to three hours. This is of little practical importance if large numbers of specimens are being rapidly examined.

* Manufactured by G. T. Gurr.

It is worth noting that anaplastic spheroidal and oat-cell carcinoma may often be found in portions of sputum which are neither purulent nor blood-stained. An apparently unsatisfactory specimen of sputum, consisting largely of mucus and debris, may in fact contain a few cells of these types of carcinoma. In contrast squamous carcinoma is more often detected in the purulent flecks.

Using methylene blue fuchsin stain the main characteristics of the non-neoplastic cells most commonly seen in sputum are as follows.

Squamous epithelium from the superficial layers of the pharynx appears as flat, rectangular, pentagonal or hexagonal forms, with small central nuclei. Cells from the deeper layers are smaller, hexagonal or circular, frequently attached to one another in small sheets or plaques, the nucleus occupying a relatively greater proportion of the cell area.

Normal respiratory epithelium is columnar, tailed on the basement membrane surface, and in well preserved specimens has a distinct brush border of cilia. The nucleus is basal (Fig. 3).

The cytoplasm of both the squamous and the normal respiratory epithelium stains a uniform bright pink or red; the nucleus is violet or purple.

Polymorphonuclear leucocytes, as in the simple methylene blue method, take up the stains capriciously unless the preparation is well mixed and warmed. Cytoplasm is a faint pink or green, the lobed nuclei a dark purple. Macrophages can be a potent source of error in the diagnosis of carcinoma. They are invariably globular, usually contain dark pigment granules which obscure the nucleus, and tend to occur in groups; the more doubtful cells can often be identified by reference to more obvious cells in the same group. Occasionally red cells or fat particles are phagocytosed. Variation in size may be extreme. The cytoplasm stains a purplish-red and the nuclei a dark purple. Suspected neoplastic cells in the neighbourhood of macrophages should be interpreted with reserve. Lymphocytes have a translucent pink or greenish cytoplasm and purple nuclei. Plasma cells have a more opaque purplish or violet cytoplasm, the oval-shaped and eccentric nucleus, typical of this cell, staining purple. Bacteria are purple or blue, and are frequently seen as a tracery in the superficial squamous epithelium. Curschmann's spirals stain an intense blue.

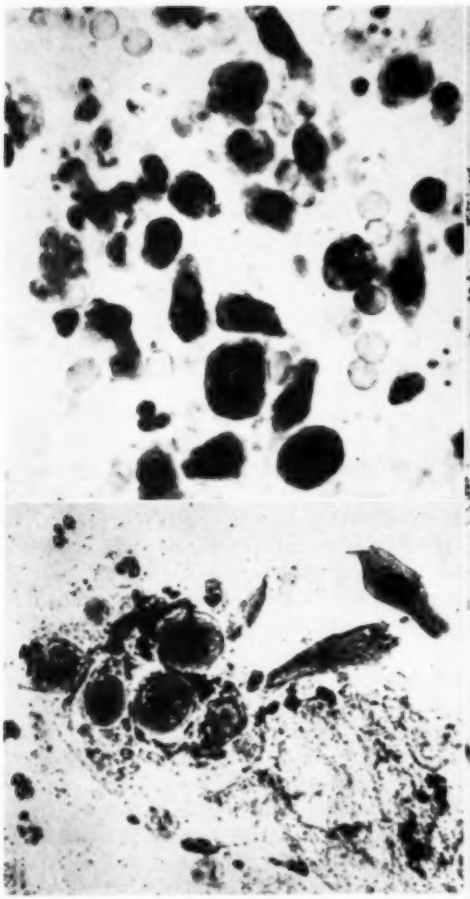
FIG. 3.—Normal respiratory epithelium. All cells similar with basal nuclei and tails, free brush border and vacuolated, foamy cytoplasm. (× 500. Methylene blue and carbol fuchsin.)

Classification of Neoplastic Cells in Sputum

Neoplastic cells in sputum can usually be classified into two main groups according to their morphology and staining reactions. The two groups are squamous-cell carcinoma and anaplastic spheroidal-cell or oat-cell carcinoma.

Squamous Cell Carcinoma.—The cells of squamous carcinoma vary enormously in size and shape (Figs. 4, 5, 6, and 7). The outline is usually irregularly oval, circular, angular or jagged with irregular processes. Sometimes signet-ring forms are seen with a large central vacuole (Fig. 7). The edge may have a doubly refractile contour. Most squamous cells contain characteristically refractile granules, sometimes forming a circle round the nucleus or dispersed throughout the cell. The keratin may be whorled, similar superficially to the Hassall's corpuscles of the thymus. The cells do not "group" so well as the anaplastic spheroidal and oat-cell types, but are scattered singly, in pairs and triads, throughout the inflammatory exudate. Occasionally larger clusters are found. The cytoplasm stains intensely, and usually irregularly, in brilliant shades of deep red in the well keratinized cells, less often violet or blue-green in the more anaplastic cells derived from the basal proliferating layers. Nuclei are purple, hyperchromatic, bizarre in shape, and frequently pyknotic. Some cells have a central space, once occupied by a nucleus. Even when embedded in leucocytes, the dense red cytoplasm and the dark purple nuclei show prominently and are far easier to identify than in the simple methylene blue preparation where the cytoplasm is a pale glistening green.

Anaplastic Spheroidal and Oat-cell Carcinoma.—In these types of carcinoma the cells are almost invariably in clumps and streaks (Figs. 8, 9, 10, and 11). In clumps they resemble clusters of grapes or frog's spawn. Individual cells may be spheroidal, oval, oat-shaped, or roughly polygonal. Individual size and shape may vary enormously in cells of a single group. This pleomorphism in one tumour is extremely common. With increasing experience it is becoming possible to grade the type of tumour more exactly in terms of predominant cell shape, rather than in the earlier broad distinction into squamous and oat-cell carcinoma that was originally employed. Where such small parts of large tumour masses are examined it is impossible to give such histologically exact descriptions as the morbid anatomist examining larger and more representative portions of tissue. Clusters of cells may be flattened by gentle pressure on the coverslip, a method which is helpful in ascertaining individual cell structure. The cells of these more anaplastic carcinomas take up the stain relatively slowly compared with normal tissues. Staining may not be complete in the groups of cells until after a period of 20 minutes to half an hour. Examined shortly after a preparation has been made, some cells will appear as refractile, glistening green disks, interspersed with others which are already stained a deep purple. All cells eventually are stained an equally dense purple, an intensity of staining unequalled by any other elements in the field. These dark groups of cells are easily identified under a 2/3 objective (Fig. 8). The purple staining is taken up by the nucleus which occupies most of the cell, the cytoplasm remaining unstained as a thin glistening rim of a pale green tint. It is of diagnostic importance that the cytoplasm practically never stains pink and never red as in the squamous cell



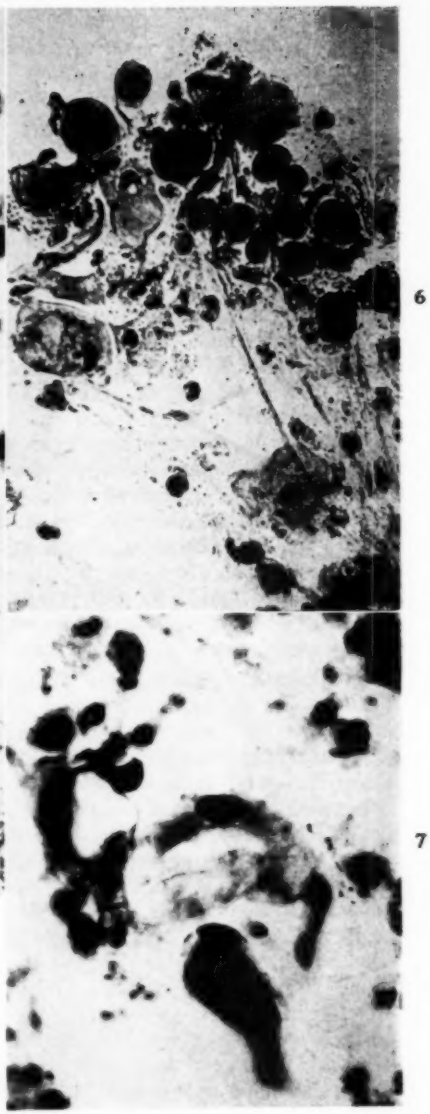


FIG. 4.—Squamous carcinoma cells. Mostly circular forms. Large hyperchromatic nuclei and densely staining cytoplasm (dark red in actual stain). A few red cells interspersed with the carcinoma cells. (×690. Methylene blue and carbol fuchsin.)

FIG. 5.—Squamous carcinoma cells. Circular forms to the left; two angular, elongated forms to the right, one with a " spinous " process, the other without a nucleus. (× 490. Methylene blue and carbol fuchsin.)

FIG. 6.—Squamous carcinoma cells. Bottom right is a normal lightly stained epithelial cell from the pharynx. Nucleus is pale. Carcinoma cells on the top right. Most have densely stained cytoplasm and all have hyperchromatic nuclei. Above and to the left of the main group are more lightly stained and better differentiated carcinoma cells, the cytoplasm irregularly vacuolated and the nuclei pyknotic. (× 370. Methylene blue and carbol fuchsin.)

FIG. 7.—Squamous carcinoma cells. Giant, bizarre forms, two of them resembling signet rings. (× 550. Methylene blue and carbol fuchsin.)

types. This has proved a most useful extra aid in the differentiation of types of growth in doubtful cases. Nuclear detail is not good, although nucleoli and chromatin networks can be distinguished in individual cells, especially before the full intensity of the stain is acquired.

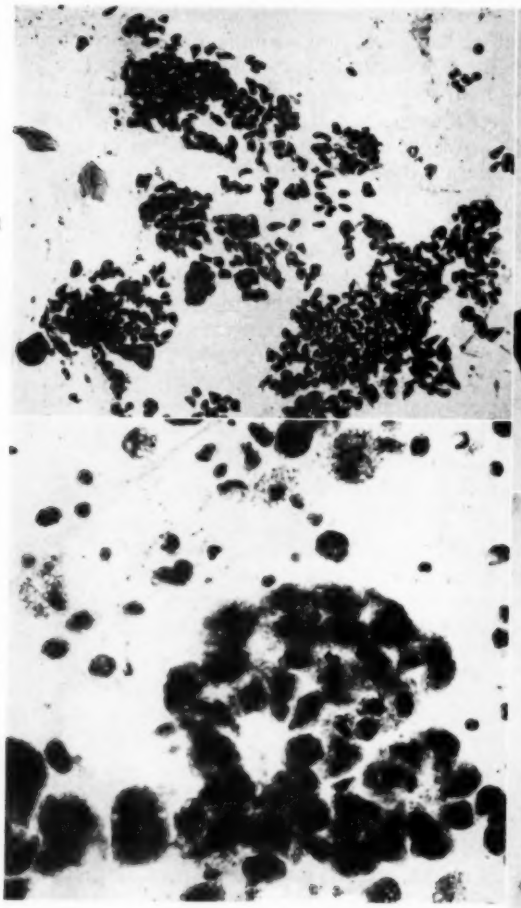
Using the iodine green stain alone, spheroidal and oat cells stain green, the nuclei being particularly distinct. Details of nucleolar structure and the pattern of chromatin filaments are visible as a darker olive green in contrast to the cytoplasm (Fig. 11). As in other methods the cells of this type are more darkly stained than normal elements of sputum, but not so darkly as to obscure the structure.

At a later date it is hoped to publish a larger series of cases examined by the above methods. A preliminary analysis has, however, already established the value of the fuchsin methylene blue stain. Using this method specimens from 174 patients have been examined where the differential diagnosis of carcinoma has been considered. A minimum of three specimens and a maximum of twelve specimens has been examined in each case, and at least three slides prepared from each specimen. Cases are completely unselected and are drawn from the wards of a large general hospital. A broad analysis of the results is appended.

The iodine green stain has been used more recently on fewer cases and requires a further probationary period, but we feel that this is the method of choice in the diagnosis of anaplastic cell carcinoma.

Total number of patients whose sputum was examined for malignant cells	174
Total number of patients diagnosed as having malignant cells in their sputum	101
Number of patients so far proved to have malignant disease of lungs by (*a*) post-mortem examination; (*b*) bronchoscopic biopsy; (*c*) drill biopsy; (*d*) histological examination of lung removed at operation	55 (100%)
Number of patients where sputum examination was positive and confirmed by the above methods	33 (60%)
Number of patients where sputum examination was negative, but other laboratory examinations were positive	22 (40%)

In addition to the cases analysed above there have so far been two false positive results. One case diagnosed as oat-cell carcinoma proved to be a tuberculoma of lung; the other, diagnosed in only one specimen as "suspicious of spheroidal cell carcinoma," was a case of disseminated lupus erythematosus. One case included in our analysis illustrates an important limitation in the interpretation of the presence of squamous carcinoma cells in sputum. Where squamous metaplasia occurs in the bronchi, as in chronic lung sepsis or over underlying malignant change, the metaplastic cells are indistinguishable from those of carcinoma. In this case squamous carcinoma was first diagnosed, and then oat-cell carcinoma on successive specimens. The biopsy report revealed "squamous metaplasia overlying an area of oat-cell carcinoma." This rare eventuality should be emphasized to any clinician who is to depend on sputum reports, which can only be ancillary to the other possible investigations. In two cases also included in our analysis, the type of carcinoma has been classified incorrectly. Both were diagnosed as oat-cell carcinoma, but one proved to be polygonal-cell carcinoma (Fig. 12), the other squamous cell. Most of those cases classified as polygonal-cell growth by the morbid anatomist fall into the group of squamous carcinoma by our method, the



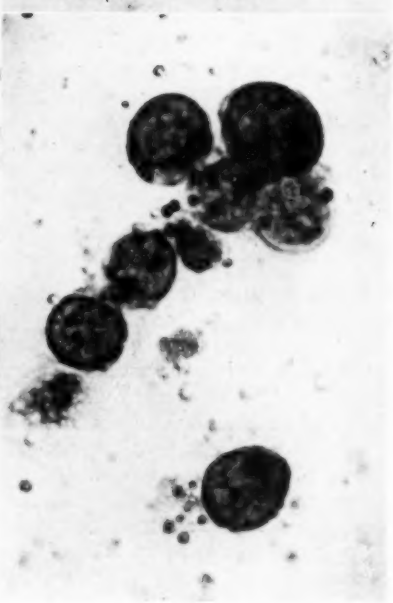


FIG. 8.—Oat cell carcinoma. Typical low power appearance of oat shaped, densely stained cells in clusters purple in actual stain. (× 115. Methylene blue and carbol fuchsin.)

FIG. 9.—Spheroidal cell carcinoma. Clusters of spheroidal cells showing hyperchromatic nuclei occupying most of cell. Scanty cytoplasm. In upper half of the picture are several macrophages with foamy cytoplasm. (× 370. Methylene blue and carbol fuchsin.)

FIG. 10.—Spheroidal and oat-cell carcinoma. Rosette of densely stained cells. Nucleolus in one cell in centre. (× 490. Methylene blue and carbol fuchsin.)

FIG. 11.—Spheroidal cell carcinoma. Spheroidal cells with distinct nuclear pattern and well stained nucleoli. Compare with Fig. 10 and notice better differentiation of nuclei. (× 1,250. Iodine green.)

FIG. 12.—Polygonal cell carcinoma. (× 385. Methylene blue and carbol fuchsin.)

cell exhibiting the characteristics already described for this type. Obviously the more anaplastic the squamous carcinoma, the more difficult is the differentiation of the type of growth from an examination of single cells or small groups of cells. Other workers using the methylene blue technique have pointed out the risk of error in specimens examined shortly after bronchoscopy. For the first week after bronchoscopy damaged cells sloughed from the respiratory tract could cause confusion in diagnosis. In occasional cases examined within this period we have experienced no particular difficulty.

We believe the figure of 60% for cases diagnosed as having malignant disease of the lung by these methods will be difficult to improve upon. Mechanical factors, such as collapse of a lobe, obstruction of a bronchus proximal to the primary growth by infiltration, or, in early stages, the failure of a growth to ulcerate, all prevent the appearance of neoplastic cells in the sputum. Similarly in the copious purulent sputum of a lung abscess secondary to a carcinoma the malignant cells are rarely detected, presumably due to dilution with the purulent exudate. For these reasons we view with some suspicion reports of a higher percentage of positive findings, unless the material were selected.

No method has so far been evolved for making permanent preparations of the already stained films, an obvious drawback in reassessing a particular case where histological technique reveals a carcinoma which has been missed in the sputum.

Summary

A method of examination of sputum for malignant cells is described, but it is emphasized that it is complementary to the other methods of investigation, bronchoscopic biopsy and drill biopsy, and in no way replaces them. The combination of all three methods is the ideal for a clinical diagnosis in the majority of cases.

We are grateful to Mr. J. King and Mr. A. L. Gallup, of the London Hospital, for the photomicrographs.

REFERENCES

Dudgeon, L. S., and Wrigley, C. H. (1935). *J. Laryng.*, **50**, 752.
Papanicolaou, G. N. (1942). *Science*, **95**, 438.
Schuster, N. H. (1947) in Dyke, S. C. (Editor) *Recent Advances in Clinical Pathology*, p. 316.

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A CASE OF ABERRANT THYROID TISSUE IN THE TRACHEA

BY

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Abberant thyroid nodules in the larynx are mentioned in standard textbooks (Price, 1941), but a study of the literature reveals that they are rare, and Joll (1932) in his book on diseases of the thyroid gland does not describe a British case.

Aberrant thyroid tissue may be formed in three ways: (1) during the process of differentiation in the foetus small fragments of primitive thyroid may be enclosed in the respiratory tract (von Bruns, 1914); (2) the developing thyroid may penetrate the wall of the larynx or trachea at some weak spot and subsequently develop in the new site (Paltauf, 1891); and (3) it is claimed that aberrant tissues may be formed as the result of neoplastic invasion of low grade malignancy. Willis (1948) considers that " lateral aberrant thyroids " should be regarded as metastatic deposits, but does not comment on central or intratracheal thyroids. He does, however, make the significant point that in the lateral examples the corresponding thyroid lobe is enlarged. Although enlargement was present in the case to be described no proof of malignancy was found and a developmental anomaly was the probable cause. This theory of origin is supported by Meyer's (1910) case in an infant at an age when thyroid neoplasm is improbable.

The first case recorded seems to be that of von Ziemssen, 1875 (quoted by Joll), in a woman 18 years old. (The majority of examples have been in women.) Dorn's (1919) case in a man, which was associated with vocal cord paralysis and occurred after local removal, would seem to illustrate Willis's (1948) contention that at least some aberrant thyroids are in fact neoplastic. Gödel (1921) published details of the case of a woman aged 39 in whom there was thyroid tissue inside the trachea joined to a hypertrophied thyroid outside the trachea.

Maier (1922) collected 28 cases, and estimated that one out of every 15 intratracheal tumours are of thyroid origin. His patient had obstructive symptoms related to pregnancy. Bundschuh (1925) met with two instances, both in women; operation for removal undertaken on account of acute occlusion of the airway in pregnancy was successful. Vacher and Denis (1927) describe a woman who gave a six years' history of respiratory distress, worse in each of four successive pregnancies. In the fourth month of the last of these she sought advice. Laryngoscopy showed a submucous tumour below the larynx and, although the Wassermann test was negative, anti-syphilitic treatment was begun but was unsuccessful. It was followed by 20 exposures to deep *x*-ray therapy, but the

symptoms became worse, and six weeks after first being seen the tumour was removed surgically. It was adhering by a broad base to the second and third tracheal rings. A living child was subsequently born at full term. This description most nearly resembles the following case.

Case Report

Mrs. C., aged 35, was first seen by a surgeon on October 1, 1943, at an antenatal examination and gave a history of periodic "asthma" of two years' duration. She had had one child 12 years before without mishap, and the second baby was due on December 23, 1943. It was noted that her respirations were stertorous, but no abnormal signs were found in the chest and her colour was good. The patient was next seen on November 3, and a breech presentation corrected by version. Stertor was again noticeable, but the chest was still apparently normal. On November 15, 1943, the patient's husband sought help urgently for her severe dyspnoea. Morphia and adrenaline brought some improvement, but her condition later grew worse and an emergency tracheotomy was performed in the patient's own home for cyanosis, apparently of obstructive origin. She was removed to hospital, and ten days later an abscess appeared close to the tracheotomy incision, and this subsequently evacuated. The abscess was assumed to be the cause of the obstruction. On December 24, 1943, a son was born without mishap, the tube was removed, and the sinus closed. The patient left hospital without stridor. She did not seek further advice till September 21, 1946, when she was again pregnant and complaining of dyspnoea. Stridor was observed, but the cause was not evident. On December 8, 1946, the patient developed an acute laryngeal obstruction, and died in the ambulance while being brought to hospital.

Necropsy.—The post-mortem examination was unremarkable except for the presence of a mass in the lower compartment of the larynx and upper part of the trachea. It

FIG. 1 (× 3.5).—Line of demarcation between thyroid outside trachea and nodule inside (considerable shrinkage has resulted from fixation).

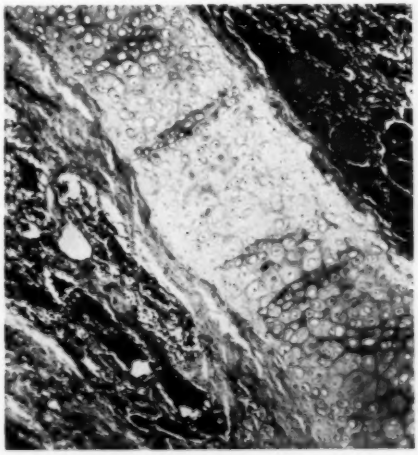

FIG. 2 (× 40).—Photomicrograph showing microscopic structure with colloid vesicles: normal thyroid to the right and above; aberrant thyroid inside cartilage to left and below.

was about ¼ in. in diameter and was adherent by a broad base on the right side from the first to the third tracheal rings. The normal mucosa was tightly stretched over the mass. When first seen the nodule was engorged and oedematous, and the lumen of the air passage was so blocked that it was only a narrow crescentic slit about one-fifth of the normal.

There was no evidence of continuity between the nodule inside and the thyroid gland outside the trachea, nor of erosion of the cartilage or soft tissue. The thyroid gland was of normal size on the left but enlarged to the right (about 2½ in. in diameter) with prominent large colloid vesicles. All the thyroid separated readily from the adjacent structures. No enlarged glands were present in the neck, but a thorough skeletal search was not made and no metastases were found elsewhere.

Histological examination confirmed the thyroid origin of the mass (Figs. 1 and 2), and demonstrated the sharp line of demarcation from the cartilage. There were large colloid vesicles, many showing recent haemorrhages. The mucosal covering was intact, and, further, the normal tracheal mucous glands persisted deep to the nodule at either edge. The adjacent right lobe of the thyroid showed a similar structure, except that haemorrhages (recent and older) were more conspicuous and there was a greater degree of acinar activity. In none of the sections was there evidence of papillary formation, blood vessel invasion, or other malignant signs.

Subsequent conversation with the husband produced the very significant statement that his wife was periodically more short of breath from Christmas, 1944, onwards and that this was always worse two or three days before the menstrual flow. This "asthma" had become progressively more severe during the next 18 months or so, but she would not seek advice as she had been alarmed by her previous tracheotomy experience. Had she complained, the true purport of the periodicity of her "asthma" might well have been appreciated.

Summary

A case of aberrant intratracheal thyroid tissue in a woman is described. This produced severe respiratory obstruction in one pregnancy and fatal asphyxia in the next.

"Asthma" occurred two or three days before each menstrual period, presumably due to cyclical changes in the thyroid nodule.

The pathology was probably due to a developmental defect and not to neoplastic metastasis.

I am indebted to Professor R. J. V. Pulvertaft for assistance with the photographs, and to Mr. Russell Stevens, F.R.C.S., under whose care the patient came at the time of her death.

References

Bruns, von — (1914). *Beitr. klin. Chir.*, **41**, 1.
Bundschuh, — (1925). *Arch. klin. Chir.*, **135**, 106.
Dorn, J. (1919). *Beitr. klin. Chir.*, **115**, 100.
Gödel, A. (1921). *Münch. med. Schr.*, **68**, 1002.
Joll, C. A. (1932). *Diseases of the Thyroid Gland.* London. Pp. 144–148.
Maier, O. (1922). *Arch. klin. Chir.*, **122**, 825.
Meyer, E. (1910). *Dtsch. med. Wschr.*, **36**, 2362.
Paltauf, R. (1891). *Beitr. path. Anat.*, **11**, 71.
Price, F. W. (1941). *Textbook of the Practice of Medicine.* 6th Ed. London. P. 1109.
Vacher, — and Denis, W. (1927). *Rev. laryng., Paris*, **48**, 761.
Willis, R. A. (1948). *Pathology of Tumours.* London. P. 610.
Ziemssen, H. von (1875). *Handbuch der spezial Pathologie und Therapie.* Leipzig. (Quoted by Joll.)

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THE AGAR-ROPE TECHNIQUE FOR ESTIMATING PENICILLIN IN LIQUIDS

BY

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Many methods for the estimation of penicillin have been published. Although chemical and physical methods may be used for its estimation in fairly pure preparations, in crude liquids and in low concentration the biological test is still necessary.

The following method is described because it can be carried out in a small clinical laboratory without special equipment and without the necessity of maintaining sterility. It is applicable to any body fluid, and it is possible to make a reading after three to four hours. The method is not suitable for the estimation of minimal detectable amounts.

Method

Serial dilutions of the material to be tested are placed in small vessels each of which contains a small block of agar containing bacteria, which, by their metabolism, produce metabolites in a short time. If glucose is added to the agar the change in *p*H is shown by an indicator such as phenol red. However, if the material to be tested shows any antibacterial activity, there will be no metabolites—i.e., no acid change. For an exact estimation of penicillin a series of dilutions of known strength are run in parallel with the test material. The results of our method were so constant that eventually we found it unnecessary to run a control series for each estimation.

Preparing the Agar Rope.—A rubber tube provided with a mouth-piece is connected to a glass tube of about 45 cm. in length by 6 mm. internal width, which is sterilized by flaming just before use or by autoclaving.

A penicillin-sensitive strain of *Staphylococcus aureus* is used. The standard Oxford strain H is suitable, but strains of different origin, when constant in their behaviour, will do as well. From an agar slope culture less than seven days old the coccus is seeded to two fresh agar slopes. The bacteria are grown at 37° for 16 hours and washed off with 2.5 ml. of sterile 0.8% saline. Both lots of bacterial suspensions are mixed and allowed to stand for sedimentation of the coarser particles. Then 1 ml. is pipetted into an ordinary test-tube and 5 ml. of glucose meat infusion broth is added. This broth must be brought to *p*H 7.6. Care should be taken that the broth has been sterilized carefully with regard to time and pressure.

Meanwhile a 4% block of agar is liquefied in water and cooled to 80° C.; 6 ml. of this is added to the 6 ml. suspension of bacteria in glucose broth, not the converse, and rapidly mixed. Then the agar mixture is drawn rapidly into the sterile 45 cm. glass tube, leaving about 1 ml. at the bottom of the test-tube, and the rubber tubing is closed with a clip. The long glass tube is quickly cooled in running tap water

without being withdrawn from the external test-tube, and the agar sets immediately. In a few minutes when the agar has solidified the long glass tube is taken out and wiped with a piece of cotton-wool. After removing the clip from the rubber tube the agar rope can easily be blown in a sterile petri dish. When the agar rope begins to form it will generally fall by its own weight. If a straight rope is preferred, it can be put in an aluminium dish 50 cm. long and covered by a lid ; in this position it will be easier to cut the rope into small blocks. For purposes of sterility the first centimetre that comes out of the glass tube is cut off and discarded. The agar rope may be put into the ice chest at once and is ready for use. In practice it has been found that the rope should not be used before the first 24 hours nor after 10 days. After use the glass tube can be cleaned easily, sterilized, and prepared for the next rope.

Making Dilutions.—For making the dilutions the same slightly alkaline meat broth with 0.5% glucose is used. To each millilitre one drop of phenol red solution (1 : 1,000) is added. It is advantageous to use a drop technique for making the dilutions. When ordinary Pasteur pipettes are standardized this technique is no less accurate than measuring by calibrated pipettes. It is easy to standardize the external diameter by moving the capillary part of Pasteur pipette in a slit of exactly 1.0 mm. width cut in a small brass plate. When more exact figures are wanted the weight of the drops of the different liquids can be determined, and the viscosity and surface tension of the different liquids thus taken into account.

Preparing Agar Blocks.—It is necessary to have agar blocks of fairly equal dimensions, and to secure equality 11 thin razor blades are joined by two long screws through their holes, and the blades separated by brass rings to a distance of 1 mm. They are then fixed to a handle. By gently pressing the knives over the rope 10 equal disks will be obtained and will adhere between the blades, from which they can be pushed down by a small spatula.

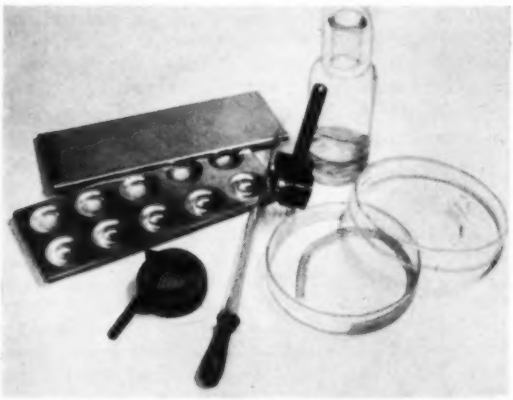

FIG. 1.—Apparatus required for agar-rope technique of estimating penicillin in liquids.

Making Titration Trays.—The tray can easily be made by impressing an aluminium plate of 2 mm. thickness with a male and female die of polished brass or iron. We used trays with 10 excavations each, each well (20 mm. diameter × 7 mm. deep) holding about 1 ml. of liquid. For practical purposes 12 drops per well is satisfactory as long as the agar disks are totally submerged. The trays are readily cleaned by rinsing. They should be well polished, as the lustre of the metal assists in reading the results. Take care to wash away traces of polish with soap or alcohol, as they may be bactericidal. It is advisable to fit some cover or lid on the trays so that they can be piled up in the incubator and evaporation is suppressed. Aluminium as a material has the advantage of low heat capacity, high heat conductivity, and relative inertness.

Carrying out the Reaction.—A penicillin solution of known strength is diluted in phenol red glucose broth to a concentration of 1/20 unit per ml. In the wells 12, 11, 10, 9, 8, 7 drops of this penicillin solution and 0, 1, 2, 3, 4, 5 drops of phenol red glucose broth are added. The wells then contain successively 1/20, 1/22, 1/24, 1/27, 1/30, 1/34 unit per ml. Then 10 agar disks are cut with the cutting device from the agar rope and one disk dropped with the small spatula into each well. (Further dilutions can be tested if desired.) The tray is covered and put into the 37° C. incubator. After three to four hours the tray can be taken out and the result read. In practice we usually found that the liquid and disks in the third well (1/24 unit per ml.) were red whereas in the fourth (1/27 unit per ml.) they were yellow. Therefore 1/24 unit per ml. suppressed the metabolic processes of the staphylococcus.

Oblique illumination should be used for taking readings. In doubtful cases the agar blocks can be lifted from the wells on to the flat surface of the trays. Artificial light makes the readings somewhat more difficult, in which case a "daylight lamp" with a blue glass bulb may be used. Readings can be made by holding the tray at the level of the lamp and inspecting vertically.

The following examples may be useful.

Estimation of Penicillin in Culture Media.—Let it be supposed that the medium contains about 70 units per ml. and then dilute it 1/1,000 in phenol red glucose broth in a 3 ml. amount. Put 12, 10, 9, 8, 7, 6, 5, 4 drops in each well successively and add 0, 2, 3, 4, 5, 6, 7, 8 drops of phenol red glucose broth from the same pipette. Suppose that in the control series suppression of acid formation again takes place in the well containing 1/24 unit per ml. If suppression in the first well only has been observed then the penicillin content of the original liquid was 42 unit per ml. It would have been 50, 56, 63, 71, 83, 100, 125 unit per ml. respectively for each successive well showing red-coloured contents. If at the same time an original dilution of 1/500 or 1/2,000 had been prepared intermediate values could have been determined, so giving a more exact reading.

Estimation of Penicillin Content of Blood Serum.—A suitable way is to add to 2 ml. of serum 1 ml. of glucose broth *p*H 7.6 without phenol red. Put 11, 10, 9, 8, 7, 6, 5, 4, 3 drops into the wells and add 0, 1, 2, 3, 4, 5, 6, 7, 8 drops of glucose broth without phenol red and then one drop of phenol red 1:1,000 to each well. It may be advantageous to prepare the standard dilution of known penicillin content with normal human serum. Suppose that again the well containing 1/24 unit per ml. of standard penicillin is the first that shows lasting red colour, then each successive red-coloured well of the unknown serum indicates an original penicillin content of 0.06, 0.07, 0.08, 0.09, 0.11, 0.13, 0.15, 0.19, 0.25 unit per ml. serum. Shortly after an intramuscular injection of 40,000 units of penicillin the blood content may be so high that a dilution of 1:10 will be necessary. Then the readings will run from 0.27 to 1.00 unit per ml. serum or even higher if necessary.

As for serum determinations, we must admit that this is not a micro-method; in our opinion, however, this is fully counterbalanced by the advantages of the very simple bacteriological demands.

Discussion

The reason why this technique works so rapidly and easily is due to the fact that it uses material very rich in bacteria. Their fixation in the agar disk protects them from external influences. In the original cylinder plate method it was known that for reproducible results it is of some advantage to keep the ready poured plates with the staphylococci for 24 hours in the ice chest. We

found that this was also true for the agar rope; we have observed too that the properties of the rope do not alter until after 10 days. The number of ropes to be prepared each week can be calculated, as we know that one rope of 45 cm. will suffice for 35 to 40 determinations. The use of material is minimal. The rich "inoculum," the fact that the indicator organism is protected by the agar, and the rapid reading make sterile precautions unnecessary. The use of clean materials and sterile phenol red glucose broth will suffice. Some chance contamination of the utensils will be of no significance whatever, as the time of incubation is four hours at the most.

In principle the use of staphylococci is not obligatory. Any other organism sensitive to penicillin and able to produce a readable change in any indicator will be suitable. It should be possible to make estimations of other antibiotics (e.g., streptomycin) by this technique. The haemolytic streptococcus is more sensitive to penicillin and it can be used in the following way.

A suspension of suitable haemolytic streptococci is prepared, preferably so that no haemoglobin can stain the fluid. This can be done by growing the streptococci on Loeffler serum medium. To 5 ml. of this suspension are added 0.75 ml. of defibrinated horse blood and 5.75 ml. 4% agar in water as already described, and the agar rope is prepared. Readings are made on haemolysis in the agar disks—i.e., the change from turbid red to laked red, and often after only two hours' incubation. In this case a standard penicillin control must be set up as the haemolytic streptococcus is much more variable than the staphylococcus. In our experience haemolytic streptococci present another disadvantage not found in staphylococci. They are, in varying degree, sensitive to the bacteriostatic power of normal serum, thus giving rise to anomalous results (Kirby and Rantz, 1944; Dolkart, Dey, and Schwemlein, 1947). More than 50% of normal sera are endowed with some anti-streptococcal power. The same is true of *Bacillus subtilis*; 90% of normal sera show some bactericidal power towards these organisms (Elias, Merrion, and Speicher, 1945). For estimations in culture media the described technique is easily adapted to *B. subtilis* (Pratt and Dufrenoy, 1947), *Streptococcus dysgalactiae*, or other organisms, all its advantages being maintained.

Summary

A technique is described for the rapid determination (after two to four hours) of penicillin or other antibiotics in liquids such as serum, body fluids, or culture media. Accurately prepared agar disks, mechanically cut from an agar rope, containing heavy inocula of bacteria, are submerged in small quantities of graded dilutions of known, compared with unknown, penicillin-containing fluids. Staphylococci are most suitable; the method can be adapted for the use of other bacteria.

An indicator (phenol red, haemolysis, etc.) detects normal compared with suppressed bacterial activity.

The method is rapid, simple, and involves the use of a minimum of utensils which are to hand day and night and so can be used with the most rudimentary bacteriological equipment. It will suffice to have the basic bacteriological material supplied weekly from a better equipped institute.

The sensitivity of the method is 0.04 unit per ml. (serum). The quantity of material wanted for the test is 2 ml. (serum of 0.04 unit per ml.) or considerably less. With streptococci sensitivity can be augmented to 0.01 unit per ml. With practice this technique will save time, personnel, and materials.

We are greatly indebted to N. V. Organon, Oss, Holland, for technical and material help.

REFERENCES

Elias, W. F., Merrion, H. J., and Speicher, T. (1945). *Science*, **102**, 223.
Dolkart, R. E., Dey, F. L., and Schwemlein, G. X. (1947). *J. Bact.*, **53**, 17.
Kirby, W. M. M., and Rantz, L. A. (1944). Ibid., **48**, 603.
Pratt, R., and Dufrenoy, J. (1947). *Nature, Lond.*, **159**, 576.

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IMPROVED PHOTOMETRIC METHODS FOR USE IN THE WASSERMANN REACTION

BY

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Strictly reproducible results can only be obtained in the Wassermann reaction if standardized procedures are followed for each step of this serological test. Methods already available for the standardization of sheep red cells and for the titration of haemolysin and complement have weaknesses which militate against either reproducibility or efficiency.

The standardization of sheep red cells has usually been performed either on the basis of the estimation of their haemoglobin content or of their number. As Orpwood Price and Wilkinson (1947) have pointed out, the sensitization of red cells by haemolytic immune antibody is a phenomenon which occurs at the cell surface. In other words, the total extent of cell surface, rather than the number of red cells or their contained haemoglobin, is the important factor in the reaction and should form the basis of any standardization procedure. As cell volume bears a direct relationship to cell surface, Orpwood Price and Wilkinson used the haematocrit to estimate the former in their attempt to overcome the objections to the procedures already in use. Though fundamentally sound, their method is time-consuming, which is always a serious disadvantage in routine Wassermann estimations.

In the titration of haemolysin and complement, 100% haemolysis is usually taken as the end-point for titre. As is well known, when the percentage haemolysis of red cells is plotted against the strength of haemolytic factor, the resultant curve is of sigmoid nature, so that the exact visual determination of the 100% haemolysis point is necessarily difficult. The various methods for the estimation of the 50% haemolysis point, which overcome this difficulty, involve the centrifuging of a partially haemolysed cell suspension and the estimation of the liberated haemoglobin. They again ignore the factor of cell surface, and introduce time-consuming steps into the estimation.

It has been found that the procedures detailed below overcome these difficulties. A little labour is involved in the standardization of these procedures, but once this is achieved their application to the Wassermann reaction shortens the time of the test considerably and produces results of remarkable constancy.

Method of Estimating Percentage by Volume of Sheep Cells Suspended in a Suitable Diluent

In the Orpwood Price and Wilkinson method of standardization of cell suspensions, the sheep cells are centrifuged for 10 minutes at 2,500 revolutions per minute, and it is

stated that by that time they are packed to a constant volume in the centrifuge in use. However, using an international centrifuge type SB, size 1, at 3,000 r.p.m. (R.C.F. at tip of tube 2,000), I found that at least half an hour was required to pack the cells contained in 2–3 ml. of suspension. This is too long for routine purposes, so the following method was evolved to reduce this time to a few minutes. It is based on the measurement of the light absorption of cell suspensions under controlled conditions. The preparation of such suspensions is first described. The study of the relationship between cell volume and transmitted light follows, and is found to conform to a mathematical formula. Finally the practical application of this for use in the Wassermann procedure is verified.

The Preparation of Cell Suspensions.—Sheep cells are collected into equal parts of Alsever's solution as given by Mayer *et al.* (1946), with the modification that 10% of 1/1,000 merthiolate is added to the suspension to inhibit bacterial growth. These cells remain stable in this fluid for many weeks when maintained at a temperature of 6° C. In agreement with Mayer *et al.* it has been found that the cells should not be used within four days of collection, after which time they react in a constant manner with haemolytic antibody and complement. Haemolysis does not occur on washing unless bacterial contamination is present.

The Preparation of the Saline Diluent.—Because the *p*H of the diluent will have an effect on the volume of the red cells, this fluid must be buffered. As, too, the Wassermann reaction consists of an interlocking series of velocity reactions sensitive to hydrogen ion changes (Richardson, 1941), its reproducibility will be improved by using buffered reagents.

Mayer *et al.* employed a saline diluent buffered with veronal and containing magnesium and calcium ions in optimal proportions to catalyse the haemolytic reaction. The whole was of strength 0.151 M and of *p*H 7.3, which is optimal for complement in the Wassermann reaction. In order to achieve these conditions the diluent is made up in the following proportions:

Sodium chloride	(A.R.) 85 g.
Barbitone	(B.P.) 6.3 g.
Sodium barbitone	(B.P.) 3.3 g.
Magnesium chloride, 6 H_2O	(A.R.) 1.01 g.
Calcium chloride, 6 H_2O	(A.R.) 0.16 g.
Distilled water to	10 litres.

A *p*H of 7.3 will usually result. This may be checked with a glass electrode *p*H meter, and if not correct the proportions of buffer salts can be altered slightly until the correct reaction is obtained. Barbitone is not readily soluble, but if the mixture is left standing overnight the slight undissolved scum on the surface goes into solution readily on stirring. Hereafter this buffered saline diluent will be referred to as saline.

The Washing of the Cells.—On theoretical grounds it is important to remove as much as possible of the adsorbed serum from the sheep cells before their sensitization by haemolytic immune antibody, for then this latter will be both even and complete. Adapting the method of Ayer, Dailey, and Fremont-Smith (1931) for the estimation of protein in cerebrospinal fluid to the analysis of protein in each wash fluid following centrifugation, I found that, with six volumes of buffered saline used at each wash, the concentration of protein in the supernatant wash fluid fell to under 1/100 of the original concentration in four washes, and decreased irregularly thereafter. Six washes are therefore used routinely as a practical compromise. After the final wash and removal of the supernatant, the cells are ready for suspension.

The Determination of the Relation between Cell Volume and Light Transmitted

A number of workers, especially in the field of photometric estimation of red cell counts, have shown that a close relationship exists between optical transmission and the number of cells present. Whitlock (1947) has demonstrated for sheep red cells that the cell shadow/volume ratio is a constant, whereas the haemoglobin concentration and cell volume vary independently within certain limits. It was therefore thought worth while to determine how transmission of light and cell percentage by volume of sheep cells were related.

Method A

Red cells from 20 separate sheep were collected and washed in the manner detailed above. Suspensions of approximately 5% were made in the diluent and their exact concentration by volume estimated by means of the haematocrit. The tube used for this purpose was made on the pattern of those used by Orpwood Price and Wilkinson. Its stem, calibrated with mercury, consisted of parallel-sided, heavy-walled "pyrex" tube 7.5 cm. in length, 7.5 mm. in diameter, and 2.1 mm. bore. It was closed at the bottom and flared at the top into a heavy open cup 4 cm. long and 1.6 cm. in diameter, and was capable of carrying 4 ml. of fluid.

Ten further exact dilutions varying from 2% to 8% were made of each standardized suspension. Of each of these 200 diluted suspensions 0.1 ml. was brought up to 6 ml. with saline and their light transmissions estimated with the Coleman No. 11 spectrophotometer at 700 μ, using the PC-5 filter.

Calculations.—The results so obtained were found by trial and error to fit the mathematical form—

$$C_v = K\left(\frac{100-T}{T}\right)^{1/n}$$

where C_v = concentration of cells by volume, T = percentage transmission, and K and $1/n$ are constants.

This, in more useful form, reads:

$$\log_{10} C_v = \log_{10} K + 1/n \log_{10}\left(\frac{100-T}{T}\right) \ldots\ldots\ldots (1)$$

For the conditions of the above experiment, the values of the constants were estimated graphically as $K = 2.04$ and $1/n = 0.825$.

Thus it is established that a relationship does exist for sheep cells between concentration of cells by volume and light transmission in such a cell suspension, and that the photometric method is one which can be employed for the estimation of such concentrations.

A disadvantage of the Coleman instrument is that the estimation of low light transmission values is inaccurate. A photometer sensitive to the lower scale of transmission would enhance the accuracy of the method. One such linear vacuum tube instrument has been devised by the author, and is described in the appendix. The constants for this, in a similar standardization to that for the Coleman spectrophotometer, but using a final volume of 5 ml. in the cuvette, were found by regression methods to be $K = 1.34$ and $1/n = 0.796$. The analysis of variance (Table I) shows that almost all the variance is due to the linear regression on concentration by volume, and that the actual error of any observation is exceedingly small. On this basis and on that of the similarity

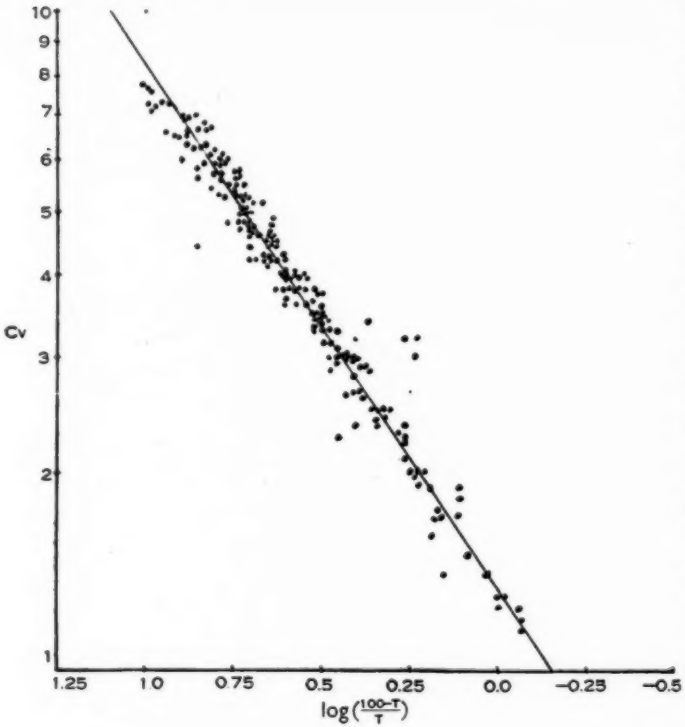

FIG. 1

between the values of 1/n for the two photometers, the second was accepted as a valid measuring instrument.

For convenience, this vacuum tube photometer was employed in all the investigations which follow. It should be emphasized that any linear photometer with a suitable light filter, modified to read low values of light transmission accurately, can be used in this standardization, and that the meter which has been described is merely one which, in practice, has proved to be very satisfactory.

In view of the fact that in the Wassermann reaction the red cells in the standard volume may show all grades of haemolysis from 0% to 100%, it was obviously necessary to find out whether the above method still held good for the determination of the volume of cells unhaemolysed in the reaction, for then it could be applied to the standardization of haemolysin and the titration of complement. In this study, the modification of the Wassermann reaction used

TABLE I

ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Mean Square	Significance
Regression	1	11.4638	Highly significant
Error	198	0.0003	

is that of Richardson (1940) and Vaughan (1947). The standard dose of red cells used, however, is 0.25 ml. of a 2.5% sensitized suspension.

Method B

A further series from 20 sheep was then prepared and each sample standardized to 2.5% by the haematocrit method. From each, nine preparations showing from 10% to 90% haemolysis were made. Of each of the 180 suspensions 0.25 ml. was diluted to 5 ml. with saline and their transmissions of light estimated in the photometer. The results were plotted on semilogarithmic paper with the percentage of unhaemolysed cells (C_p) on the logarithmic axis and $\log_{10}\left(\frac{100-T}{T}\right)$ on the arithmetic axis, and the points again fell on a straight line. Therefore this method of estimating cell percentage by volume holds good under these conditions.

Calculations.—The constants, when applied to the formula

$$\log_{10} C_p = \log_{10} K + 1/n \log_{10}\left(\frac{100-T}{T}\right) \ldots\ldots\ldots (2)$$

where C_p = percentage by volume of cells unhaemolysed in the standard volume, were found by regression methods to be K = 18.527 and 1/n = 0.907. The analysis of variance shows similar features to the previous one. The increased value of the constant 1/n is due to the presence of the liberated haemoglobin.

TABLE II
ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Mean Square	Significance
Regression	1	19.3045	Highly significant
Error	178	0.0036	

To make these relationships available for the routine Wassermann reaction, two tables must be constructed using equations (1) and (2). The first relates transmission percentage to percentage by volume of sheep cells suspended in saline (C_v), and is used in the routine standardization of sheep cells. The second relates transmission percentage to percentage of standard cell volume, unhaemolysed (C_p), and is used in the titration of haemolysin and complement.

The Standardization of Haemolysin and the Titration of Complement

In the titration of haemolysin and complement, 100% haemolysis is often taken as the end-point. This is likely to be inaccurate. Other workers, such as Wadsworth, Maltaner, and Maltaner (1931) have used the 50% haemolysis point for standardizing these substances using the formula attributed to von Krogh

$$X = K\left(\frac{Y}{1-Y}\right)^{1/n} \ldots\ldots\ldots (3)$$

where X = the amount of complement causing the haemolysis of a fraction Y of the cells sensitized with specific immune antibody, K and 1/n being constants.

In their methods, the fractions of cells haemolysed are determined in the reaction tubes by first centrifuging the latter and then estimating the liberated haemoglobin. This procedure has the disadvantage of being time-consuming, and is theoretically unsound as the factor of cell surface is ignored.

It has been shown that cell volume can be estimated directly in the presence of haemolysis. To make this relationship available for the above titrations equation (3) has to be modified in the following manner. If a fraction Y of cells are haemolysed, they are related to C , the percentage of cells remaining thus

$$C_p = 100(1-Y) \text{ or}$$

$$Y = \frac{100-C_p}{100}$$

so that on substituting for Y in equation (3) it becomes,

$$X = K\left(\frac{100-C_p}{C_p}\right)^{1/n} \ldots\ldots\ldots\ldots (4)$$

The use of this equation by-passes the objections to the methods which are based on the use of equation (3).

The Standardization of Haemolysin.—Many authors—for example, Kent (1947)—have found that, with increasing doses of haemolysin, the value of 1/n in equation (3) falls to a minimum and then increases. They have used the amount of haemolysin corresponding to this minimum as the dose for the Wassermann reaction. In the present work it was found that, for haemolysis times of 15 minutes in a water bath at 37° C., using 0.25 ml. of 2.5% sensitized cells in the presence of graded amounts of complement, the minimum value of 1/n was 0.2. However, the calculations involved in the determination of this value are much too complicated to use for routine purposes, as they have to make use of graphical constructions, or of mathematical analysis by mean squares (Kent *et al.*, 1946) or probits (Fisher, 1948).

Wyler's (1929) method of estimating the minimal haemolytic dose of haemolysin for use in the Wassermann reaction is in essence simple and easily carried out. However, the sigmoid nature of the haemolysis curve and the visual estimation of 100% haemolysis make the end-point difficult to determine with precision. I have therefore modified the method by making use of the technique of estimating cell volume described earlier, and thereby putting this determination on an exact basis. The sample of haemolysin to be standardized is titrated according to the method of Wyler, but using 2.5% cells standardized as above. The results obtained in the photometer are plotted on semilogarithmic paper, the volume of cells haemolysed derived from equation (2) and its table, on the logarithmic axis, and the concentration of haemolysin on the arithmetic axis. This results in the expected sigmoid curve. The straight line portion of this is extrapolated to 100% haemolysis, and the concentration of haemolysin corresponding to this point taken as the minimal haemolytic dose.

Taking, as Wyler did, six times the minimal haemolytic dose for use in the Wassermann reaction, it was found that this corresponded exactly to the amount of haemolysin required to make the factor 1/n take the value 0.2 in equation (4). As will be shown later, this factor can be checked automatically during the routine titration. Therefore this simple procedure of titrating haemolysin is justified, because it has been shown to be accurate and at the same time avoids the necessity for complicated graphical or mathematical analyses.

The Preparation of Sensitized Sheep Cells.—Sheep cells are washed and made up in saline to a strength of 5.5% to 6% by volume and their exact percentage estimated according to the standard procedure described and by reference to the tables derived from equation (1). The amount of this suspension required to be diluted to form

a 2.6% suspension in 100 ml. saline is placed in a wide mouthed 250-ml. bottle. Haemolysin in the correct dose, determined as described, is added to the volume of the saline required to make the final 100 ml. In order to ensure even sensitization, this latter is added in one movement to the cell suspension while it is being swirled in the 250-ml. bottle. The concentration by volume of cells in this final suspension (C_v) is estimated as before and adjustment made, if necessary, to bring the cell volume to 2.5%. A 2.6% suspension is prepared in the first place, as it is always easier to dilute to the final figure than to concentrate to it. This 2.5% sensitized suspension is placed in the water bath at 37° C. for 15 minutes and is then ready for use in the later stages of the Wassermann reaction.

The Titration of Complement.—The estimation of cell volume (C_p) using the 50% point is used as the basis for the standardization of a method for the titration of complement. The method of Kent *et al.* (1946) for 50% haemolysis has been adapted to the estimation of cell volume and gives particularly good results. However, it is a graphical method and it was thought that, for the sake of uniformity and slightly greater accuracy, the following alternative mathematical approach merited attention.

It has been shown in equation (4) that in a complement titration the amount of complement can be related to the cell volume. It has also been shown that the factor $1/n$ can be stabilized at 0.2, so that we can now write equation (4) thus

$$X = K\left(\frac{100-C_p}{C_p}\right)^{0.2} \quad \ldots\ldots\ldots (5)$$

Now let

$$\left(\frac{100-C_p}{C_p}\right)^{0.2} = Z \quad \ldots\ldots\ldots (6)$$

then $X/Z = K$

On examining equation (5), it is seen that if $C_p = 50$ (that is, if 50% of the cells by volume in the haemolytic reaction remain unhaemolysed), $X = K$. This means that if the value of K can be estimated, the dose of complement required for use in the Wassermann reaction can be defined exactly. The estimation of this constant is achieved by carrying out a titration of complement and determining the values of Z (equation 6) for each value of X. The quotient X/Z is then K, and is recorded for each tube of the titration.

The standard method used for titrating complement, which is, in fact, a modification of Wyler's technique, is now described in detail, as the values of X and Z will vary according to the quantities of the reagents used and the conditions under which they are allowed to react.

Preparation of Complement.—Twelve exsanguinated guinea-pigs provide each pool of complement. This is dosed into 5 ml. quantities in 4 in. by ½ in. test-tubes, corked and stored at −16° C. In this state the complement has been shown to be stable for many weeks. When required for use, a tube is thawed on the bench and the complement diluted to 50 ml. with saline, thus providing a 1 in 10 dilution. This is kept at 6° C. until actually required for use.

Method for Titration of Complement.—Two rows of ten 4 in. by ½ in. test-tubes are set up in a rack and saline is pipetted into the back row, proceeding from left to right in the series 2.6, 2.8 ..... 4.4 ml. Into the front row is pipetted 0.5 ml. of saline. Into each tube of the back row is now pipetted 0.25 ml. of 1 in 10

complement in saline and, beginning at the tube with the greatest dilution and working down to that with the least, the contents are well mixed. Then 0.25 ml. is transferred with a clean pipette from each of these tubes to the corresponding front one. The rack is warmed for two minutes in the water bath at 37° C., and 0.25 ml. of the standardized sensitized cells, also warmed to 37° C., are added to each tube of the front row. These are shaken briefly at the time of addition to ensure even distribution of complement among the red cells. The complement should not be warmed for more than two minutes because of its instability in high dilutions of serum. When the addition of cells is complete, the rack is shaken two or three times and returned to the water bath for 15 minutes, during which time it is removed and shaken again on two occasions. When incubation is complete 4 ml. of saline are added to each tube of the front row and the light transmissions determined for each suspension.

Calculation of Factors.—The values of X for each of the 10 tubes are calculated as follows. Let R be the volume of saline in ml. initially present in any tube of the back row. Then the amount of complement, X, in each of the front tubes will be,

$$\frac{0.25 \times 0.25}{10\,(R + 0.25)} \text{ ml.}$$

Now, while knowledge of this quantity of complement is essential in theoretical calculations, for routine purposes the total amount of complement to be made up for any session is required to be known. For convenience, this can be expressed as the volume of 1 in 10 complement which has to be diluted to 100 ml. so that 0.25 ml. will contain one 50% dose. It will be readily appreciated that the relationship between the amount of complement in the front tubes of the rack (the actual titration row) and the 100 ml. of final complement is as 1:4,000, i.e.

$$X = \frac{25}{R + 0.25}$$

Thus the values for X for each of the ten tubes can be tabulated.

To avoid the labour of calculating the values of Z from equations (2) and (6) for each light transmission figure T whenever complement is to be titrated, a table relating T to Z is prepared. The comprehensive tables of Ecker and Hiatt (1949) may be used for values of the function $\left(\frac{Y}{1-Y}\right)^{1/n}$, for as $C_p = 100\ (1 - Y)$, these tables apply to the formula $\left(\frac{100-C_p}{C_p}\right)^{1/n}$ also.

In view of the fact that the value of 1/n in equation (6) is 0.2, and that C_p can be calculated for each value of T from equation (2), a table of Z can be prepared for each possible value of T. From this the actual values of Z for the light transmission figures in the complement titration are at once obtained.

For the relation $K = X/Z$, ten separate values of this constant can now be obtained. This represents ten separate estimates of the 50% unit of complement. On comparing the value of the unit of complement standardized by the original Wyler method with that of the 50% unit derived as above, it was found that three Wyler units were approximately equal to eight 50% units. Hence on adding the values of K for any successive eight out of ten tubes, the amount of 1 in 10 complement required to be diluted to 100 ml. to give eight 50% complement units is obtained. The eight values of Z are selected to be as near mid-range as possible.

TABLE III
TABLE OF TITRATIONS

R	X	Z	K
2.6	8.8	1.34	6.6
2.8	8.2	1.26	6.5
3.0	7.7	1.25	6.15
3.2	7.25	1.22	5.9
3.4	6.85	1.09	6.2
3.6	6.5	1.12	5.8
3.8	6.2	1.09	5.7
4.0	5.9	1.08	5.4
4.2	5.6	1.00	5.6
4.4	5.4	0.95	5.7

Table III gives a worked example of an actual titration using this method.

Adding the values of K for tubes R 3.0 to R 4.4, inclusive, gives the value 46.45. This is the amount in ml. of the 1 in 10 complement which, when diluted to 100 ml. with saline, will contain eight 50% units in 0.25 ml.

An additional reason why this method of estimating the 50% unit of complement is valuable is that it can be seen by inspection if the factor $1/n$ determining the slope of the complement titration line is approximately 0.2. If it is not, the factor K will vary continuously, instead of around a central figure, indicating that the haemolysin needs re-titrating.

This method, when applied to the Richardson-Wyler modification of the Wassermann reaction, has proved satisfactory in routine use over the past year, during which time approximately 10,000 sera have been examined in daily sessions. It has been responsible for a great increase in speed and accuracy in performance of the test.

It makes use of the estimation of cell volume and so takes account of cell surface, thereby placing the estimations on a sound theoretical basis. The method involves the reading, in a photometer, of light transmission values of sheep cells suspended in a suitable diluent, and the obtaining of certain figures by reference to four tables. From these, by simple calculations, the sheep cells, haemolysin, and complement may be standardized exactly and in the matter of a few minutes.

Some indication of the accuracy of the standardization procedure in given by the fact that the value of $1/n$ in 20 unselected, routine, serial complement titrations was calculated by the method of mean squares and was found to be 0.206 ± 0.017. The rapidity of standardization makes well worth while the initial labour involved in the preparation of the tables. Finally, the method has the great advantage that a continuous check can be kept on the activity of both haemolysin and complement without any additional labour being involved.

Summary

Improved photometric methods for the standardization of sensitized sheep cells and haemolysin and for the titration of complement for use in the Wassermann reaction are described. They involve the measurement of red cell volume, which is considered the factor most closely related to the cell surface, the point at which the haemolytic reaction occurs.

In the interests of accuracy and speed these methods are recommended for adoption in the routine performance of this Wassermann reaction.

I wish to thank Miss N. R. L. Davies, B.Sc., for her willing and able technical assistance during this work, Dr. J. L. Pinniger for his helpful criticism and advice, and Mr. H. R. Thompson, M.Sc., of the Applied Mathematics Laboratory, Wellington, for help with the statistical analyses.

References

Ayer, J. B., Dailey, M. E., and Fremont-Smith, F. (1931). *Arch. Neurol. Psychiat., Chicago*, **26**, 1038.
Ecker, E. E., and Hiatt, C. W. (1949). *Amer. J. clin. Path.*, **19**, 141.
Fisher, J. W. (1948). *J. Immunol.*, **60**, 85.
Kent, J. F. (1947). *Science*, **105**, 316.
—— Bukantz, S. C., and Rein, C. R. (1946). *J. Immunol.*, **53**, 37.
Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M. (1946). *J. exp. Med.*, **84**, 535.
Morgan, R. H., and Weinhouse, S. (1940). *J. Lab. clin. Med.*, **26**, 416.
Orpwood Price, I. N., and Wilkinson, A. E. (1947). *Brit. J. vener. Dis.*, **23**, 124.
Radiotronics. Technical Bulletin No. 109 (1941). Amalgamated Wireless Valve Company, Sydney.
Richardson, G. M. (1940). *Brit. J. vener. Dis.*, **16**, 166.
—— (1941). *Lancet*, **2**, 696.
Vaughan, A. C. T. (1947). *Brit. J. vener. Dis.*, **23**, 77.
Wadsworth, A., Maltaner, E., and Maltaner, F. (1931). *J. Immunol.*, **21**, 313.
Whitlock, J. H. (1947). *Blood*, **2**, 463.
Wyler, E. J. (1929). *Spec. Rep. Ser. med. Res. Coun., Lond.*, No. 129.

A BRIDGE CIRCUIT, VACUUM TUBE PHOTOMETER

The circuit diagram (Fig. 2) of the pentode, bridge balance photometer which was used in the photometric methods described is self explanatory. It is similar to one in a *Radiotronics Bulletin* (1941) and uses some of the constructional ideas of Morgan and Weinhouse (1940).

The type 926 R.C.A. photocell was used as it was on hand, but no doubt other types, especially those sensitive in the red part of the spectrum, would be as satisfactory. It will be noticed that the 0–50 microammeter is normally shunted so that it is in effect a 0–250 microammeter, thus preventing accidental overload of the meter. On pressing the "multiplier key," it reverts to its original range, thus making it simple to read values of transmission under 20%, which are thereby multiplied by a factor of 5. The meter still remains linear over this part of the scale.

In wiring, normal amplifier practice was followed, all earthing being brought back to a common point. The wiring was with P.V.C. cable which completely stopped any instability due to leakage. The screened phototube was mounted on a polystyrene block immediately above the input grid. The grid load resisters and their associated condensers were wired direct to the earthed valve shields.

The Coleman square cuvette was used and was illuminated by a small dental bulb with fused-in lens. This was mounted almost in contact in the wall of the cuvette well. Between the cuvette and the photocell was mounted an Ilford tri-colour red filter which has a density of no more than 0.1 at wavelengths over 620 μ, thus minimizing the effect of haemoglobin on the overall transmission.

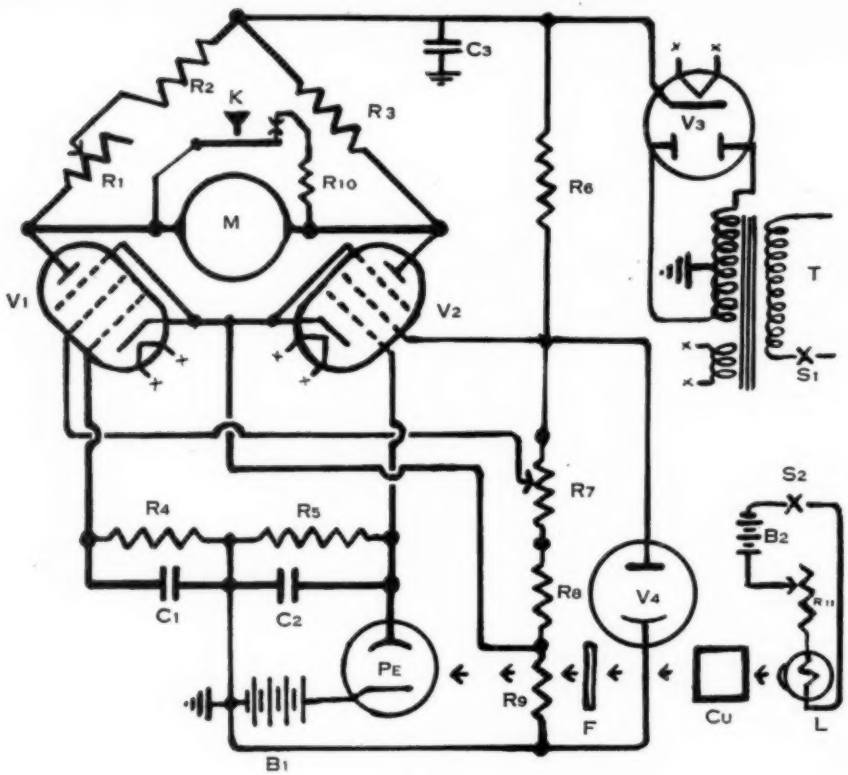

FIG. 2

B1 = 22.5 V. miniature dry battery.
B2 = 3 V. heavy duty dry battery.
C1, C2 = 0.01 mfd. mica condenser.
C3 = 8 mfd., 450 V. working, electrolytic condenser.
Cu = Cuvette.
F = Ilford tri-colour red filter.
K = "Multiplier" key ("push to talk" switch).
L = Dental torch bulb with fused-in lens.
M = 0–50 microammeter.
PE = Type 926, vacuum phototube.
R1 = 1,000 ohms, 1 watt.
R2, R3 = 50,000 ohms, 1 watt.
R4, R5 = 10 megohms, ½ watt.
R6 = 2,500 ohms, 2 watts.
R7 = 10,000 ohms, 1 watt.
R8 = 2,500 ohms, 1 watt.
R9 = 300 ohms, 1 watt.
R10 = X 5 shunt to suit meter.
R11 = 20 ohms filament rheostat.
S1, S2 = On/off switch.
T = Power transformer, 230 V.A.C. primary; 150,0,150 V., 40 mills.; 6.3 V., 2 A. secondaries.
V1, V2 = Type 6 J 7-G valve.
V3 = Type 6 X 5-GT valve.
V4 = Type V R 105–30 voltage regulating valve.

J. clin. Path. (1950), 3, 67.

A CENTRIFUGAL FILTER FOR STERILIZING SMALL AMOUNTS OF FLUID

BY

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It is often desirable in routine and experimental laboratory work to obtain sterile filtrates of small quantities (1–5 ml.) of liquid, and the principal disadvantage of the usual types of bacterial filter is the relatively large loss by absorption in the filtering element. To overcome this difficulty Morton and Czarnetzky (1937) used a funnel fitted with a sintered (fritted) glass disk of 30 mm. diameter, porosity "5 *auf* 3," the capacity being 30 ml. This funnel was fitted with a rubber stopper into the mouth of a large-bore test-tube having a side-arm to which suction was applied. Asheshov (1938) described a smaller piece of apparatus consisting of a small Chamberland L_3 or L_5 filter-candle fitted to the lower end of a 10–20 ml. pipette which had a rubber bulb or suction lead at its upper end. The candle was immersed in the fluid to be sterilized and suction applied.

The filtration pressure resulting from centrifugation may be used in a clarifying and sterilizing filter, and Boerner (1942) described a metal filter for use with ordinary laboratory centrifuges. This is made up of two parts: a base holding a Seitz filter pad, and a cylindrical upper part threaded at its lower end. It is assembled by screwing down the top cylinder tightly on to the filter pad and the filter is then fitted into the top of a standard metal centrifuge tube and spun at 2,000 revolutions per minute. When filled to its capacity of 2 ml. with, for example, serum, filtration is complete in 10 to 15 minutes with a loss of about 0.3 ml. (15%). Less viscid fluids filter more rapidly and the loss is smaller. This filter is a marked advance, in principle and design, on the two types mentioned above, and the loss of fluid is minimal. There are, however, certain disadvantages: the filter and its container are sterilized apart, with the risk of contamination; a direct rotational shearing force is applied to the filter pad, with the likelihood of a disturbance of structure and unreliability in use; and the capacity is too small in relation to the filtration area and overall dimensions.

The filter described here (see Fig. 1) follows that of Boerner in principle, and consists essentially of a flanged metal tube with its upper part separated by a Seitz EK pad from the lower, the overall dimensions allowing of its use in standard laboratory centrifuges. In some centrifuges an adapter collar may be required to prevent the filter from touching the outer casing. The upper portion, or cup, into which the fluid to be filtered is placed has a capacity of 5 ml. It has a flange like an ordinary centrifuge bucket and a pair of facets on which a spanner may grip. Its lower part carries an outer thread and tapers to a pressure ring, which, when the filter is assembled,

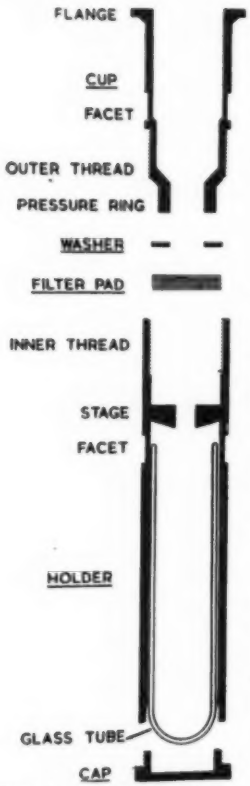

FIG. 1.—Diagram of the filter, the actual measurements of which are 4½ in. high by ¾ in. diameter.

is separated from the filter pad by a metal washer. The lower section of the apparatus has at its upper end a deep internal thread, a similar pair of facets for a spanner, and a stage on which the filter pad rests. The stage has a hole through the centre so that the drops of filtrate are directed down the central axis. Below the stage the holder contains a glass tube to receive the filtrate and is closed at its lower end by a screw cap knurled for tightening by hand.

In use, the holder is first loaded with the glass receiving tube, and the filter pad is placed on the stage, followed by the washer. If pads of suitable size are not available, they may be cut out of larger well-tested Seitz filter pads. With the aid of a pair of spanners the cup is then screwed down firmly into the top of the holder, thus tightly compressing the pad between the stage and the washer. The leverage of the spanners is needed to give a greater force than that obtained with a milled surface by hand. The assembled filters are sterilized in pairs in the autoclave. Filtration is complete in 15 to 25 minutes at about 2,500 r.p.m., depending on the viscosity and volume of the fluid, and the loss by absorption is not more than 0.4 ml. (8% of 5 ml.). For safety, the cup may be corked with a rubber stopper without loss of efficiency.

This design was found reliable and handy. Its advantages are (*a*) that the rotational shearing strain communicated to the pad is negligible, since the pressure ring turns freely on the smooth upper surface of the washer, and the reliability of the pad is not endangered; (*b*) the avoidance of the risk of contamination of the receiver, since the apparatus is made up and sterilized as a unit; and (*c*) the fluid loss is minimal.

The versatility of the filter is increased by the provision of a base with a side-arm for suction, on to which the uncapped holder may be screwed at an air-tight joint, so converting the filter from the centrifugal to the normal suction type. Used in this way, a narrower, longer glass tube receives the filtrate, and the total capacity is increased.

Summary

A centrifugal bacterial filter is described, suitable for small quantities of fluid, and possessing the advantages of minimal percentage loss by absorption, ease of handling, avoidance of contamination of the filtrate, and convertibility to a suction type of filter of slightly larger capacity.

The filter is obtainable from Messrs. M. S. E., Ltd., 14, Spenser Street, London, S.W.1.

REFERENCES

Asheshov, I. N. (1938). *J. Bact.*, **36**, 197.
Boerner, Fred (1942). *Amer. J. clin. Path.*, **12** (Tech. Sect.), 68.
Morton, H. E., and Czarnetzky, E. J. (1937). *J. Bact.*, **34**, 461.

J. clin. Path. (1950), 3, 69.

THE DAILY EXCRETION OF RIBOFLAVIN, BIOTIN, PANTOTHENIC ACID, AND NICOTINIC ACID DERIVATIVES BY NORMALS

BY

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(RECEIVED FOR PUBLICATION, NOVEMBER 8, 1949)

Cases of marked vitamin B deficiency are not infrequently admitted to hospital. Such usually show improvement when treated with preparations containing most of the members of the vitamin B complex. Clinical signs alone often make it difficult to assess whether a particular member or a number of the members of the vitamin B complex are involved. Clinical evidence would suggest that, in many cases, a number of the members of the vitamin B complex are involved. It is possible that laboratory investigations in conjunction with clinical evidence may help to throw some light on this problem. Members of the vitamin B complex exist in biological fluids in low concentrations, and for the majority there exist few or no suitable chemical methods for their determination. The recent development, however, of microbiological assay techniques has now opened up possibilities for laboratory investigations. Fitzpatrick and Tompsett (1949) have described microbiological techniques suitable for the determination of riboflavin and nicotinic acid concentrations in urine. In the present investigation the excretion of these substances and of biotin and pantothenic acid in the normal man is described. The investigation was made because in certain of them, e.g. nicotinic acid, there are wide variations in the normal ranges and most of the determinations have been carried out in the U.S.A. where dietary conditions are different.

Riboflavin appears to be excreted in urine in the free state and so far no other excretory product has been detected. Available figures, which appear to be mostly from American sources, suggest that in the normal man about 500–1,000 μg. are excreted per day (Bicknell and Prescott, 1946).

Both nicotinic acid and nicotinamide, which are microbiologically active towards *L. arabinosus* 17/5, are excreted in urine. Ranges for these, as quoted by Bicknell and Prescott (1946), vary extremely widely. Recently it has been shown that nicotinic acid is excreted mainly as two microbiologically inactive metabolites: N_1-methylnicotinamide (Huff and Perlzweig, 1945), and the 6 pyridone of N_1-methylnicotinamide (Knox and Grossman, 1946 ; 1947). Using a fluorimetric method, Huff and Perlzweig (1947) have found that normal human urine contains 3–17 mg. per day of N_1-methylnicotinamide.

Biotin appears to be excreted in urine in the free state, and so far no other excretory product has been detected. Available figures, which are few and are of American origin, suggest that in the normal man about 14–111 μg. are excreted per day (Bicknell and Prescott, 1946).

Pantothenic acid appears to be excreted in urine in the free state, and so far no other excretory product has been detected. Available figures, which also appear to be from American sources, show that in the normal man about 1.5–6.8 mg. are excreted daily (Bicknell and Prescott, 1946).

Experimental Method

Urine was collected from 37 persons on a normal diet. Specimens were collected for 24 hours in dark brown Winchester quart bottles containing 5 ml. of glacial acetic acid, and were thereafter preserved in a refrigerator.

The following assay methods were employed:

Riboflavin, microbiologically with *L. casei* (Fitzpatrick and Tompsett, 1949).

Nicotinic acid, microbiologically with *L. arabinosus* 17/5 (Fitzpatrick and Tompsett, 1949).

N_1-methylnicotinamide, fluorimetrically by the method of Huff and Perlzweig (1947).

Biotin, microbiologically with *L. arabinosus* 17/5 by a modification of the method of Barton-Wright (1945).

Pantothenic acid, microbiologically with *L. arabinosus* 17/5 by a modification of the method of Barton-Wright (1945).

The results of the assays are shown in Table I.

TABLE I

DAILY EXCRETION OF RIBOFLAVIN, NICOTINIC ACID, N_1-METHYLNICOTINAMIDE, BIOTIN, AND PANTOTHENIC ACID BY 37 NORMALS

Vitamin	Minimum	Maximum	Mean	Standard Deviation
Riboflavin (μg.)	65	980	345	217
*Nicotinic acid (μg.)	295	2,000	738	313
N_1-methylnicotinamide (mg.) ..	2.0	12.5	5.8	2.6
Biotin (μg.)	5	20	13	4.2
Pantothenic acid (mg.) ..	2.5	9.6	5.7	2.1

* Includes all nicotinic acid derivatives (nicotinic acid, nicotinamide, nicotinuric acid) microbiologically active towards *L. arabinosus* 17/5.

The figures for the excretion of riboflavin show a much wider range than those from the U.S.A., particularly at the lower levels. Figures for biotin excretion are found to be towards the lower end of the American range. Figures for pantothenic acid and N_1-methylnicotinamide excretion are very similar to the American ranges.

Summary

Figures for the excretion of riboflavin, nicotinic acid, N_1-methylnicotinamide, biotin, and pantothenic acid by 37 normal persons are presented.

References

Barton-Wright, E. C. (1945). *Analyst*, **70**, 283.
Bicknell, F., and Prescott, F. (1946). *The Vitamins in Medicine*. Heinemann. London.
Fitzpatrick, J., and Tompsett, S. L. (1949). *J. clin. Path.*, **2**, 121.
Huff, J. W., and Perlzweig, W. A. (1945). *J. biol. Chem.*, **150**, 395, 483.
—— —— (1947). Ibid., **167**, 157.
Knox, W. E., and Grossman, W. I. (1946). Ibid., **166**, 391; **168**, 363.

J. clin. Path. (1950), 3, 72.

TECHNICAL METHODS

A SIMPLE AND PRECISE TECHNIQUE FOR THE TITRATION OF HAEMAGGLUTININS

BY

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From the Istituto Sieroterapico Italiano, Naples

(RECEIVED FOR PUBLICATION, JULY 23, 1949)

The need for a standard technique for the titration of haemagglutinins has frequently been stressed. There is little doubt that among the causes of error in this test the difficulty of evaluating exactly the degree of agglutination and thus the endpoint of the reaction is the most important (Carlinfanti and Cavalli, 1947). A simple technique has therefore been designed (Carlinfanti and Molla, 1947), the essential feature of which, as in the Hirst method (1942) for virus titration, is the comparison of the sedimentation rates of agglutinated and unagglutinated red cells.

The test can be read objectively without special equipment by observing the column of red cell suspension at a fixed interval of time (four hours) after mixing it with the serum dilutions and comparing it with a standard tube prepared by diluting the same suspension. By means of a number of investigations on the influence of temperature, time, concentration of erythrocytes, etc., the procedure has been developed to give the maximum accuracy and simplicity of performance.

Technique

Small test-tubes of an internal diameter of 8 mm. and approximately 7 cm. long are used ; in the rack they should be held vertically in order to prevent red cells from clustering unevenly at one side of the tube. An empty hole is left between each tube and the next, so that the standard can be placed beside any tube during the reading of the test. Serial twofold dilutions of the serum are prepared in 1 ml. saline and then 1 ml. of a 1% suspension of washed red cells* is added with a thin 1-ml. pipette in such a way that by expelling the liquid from the pipette into the tube the suspension is well mixed with the diluted serum.

The tubes are now set aside at ordinary room temperature (between 17° and 25° C.) for exactly four hours. In the meantime a standard tube is prepared by

* This suspension can be standardized by checking with haematocrit the density of a more concentrated suspension and correcting the error in carrying out further dilution to 1%. But this correction does not seem to be necessary, since errors of up to $\pm 50\%$ of red cell density do not affect appreciably the result of the test, provided, of course, the standard is prepared from the same suspension used for the test.

adding three volumes of saline to one volume of the suspension used for the test; 2 ml. of this 0.25% cell suspension are placed in a tube and carefully shaken just before use. The reading is performed by comparing its colour and opacity with those of the intermediate zone of the column of liquid contained in the various tubes of the titration. A paper sheet with a rectangular hole 1 cm. high and some 5–6 cm. long will be used for hiding the upper part of the liquid, which is clear, and the lower part of the tube where the sediment has settled (Fig. 1).

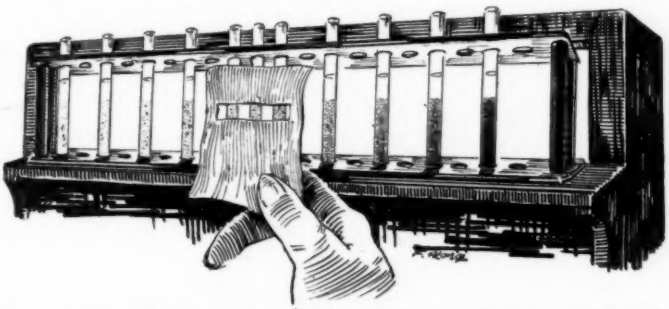

FIG. 1.—Diagram showing method of reading titres by the use of a standard tube.

The endpoint of the titration is that at which 50% of the cells involved remain suspended after four hours of settling. Accordingly the titre assigned to the serum corresponds to that dilution giving a cell density equal to the standard in the middle part of the tube. In case none of the tubes corresponds to the standard, the titre will be determined by interpolating one or two values between two twofold dilutions.

This technique has been in use in our own and other laboratories for over two years and has proved to be very satisfactory. Its precision is far greater than that of the usual technique in which the reading is performed by observing the presence and size of the red cell aggregates; it can be further enhanced, if necessary, by adopting a photoelectric colorimeter. The reciprocal of the titre obtained can be considered as the number of "haemagglutinating units" contained in 1 ml. of the serum on test. This method, however, does not obviate the need for standard sera to which this arbitrary unit can be referred. In the titration performed by this method A_2 red cells give markedly lower titres than A_1 cells. Furthermore, by measuring with appropriate standard suspensions the cell density in the tubes where the serum concentration is higher than the endpoint, a curve can be drawn, whereby the agglutinating properties of the serum can be more exactly featured (Grifoni, 1948).

REFERENCES

Carlinfanti, E., and Cavalli, L. L. (1947). *Riv. Ist. Sieroter. Italiano*, **22** (N.3), 129.

—— and Molla, W. (1947). *Un metodo preciso e semplice per la titolazione delle emoagglutinine.* Congrès international de la transfusion sanguine, Turin.

Grifoni, V. (1948). Personal communication.

Hirst, G. K. (1942). *J. exp. Med.*, **76**, 195.

J. clin. Path. (1950), 3, 74.

MICROTECHNIQUE FOR BLOOD CULTURES

BY

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(RECEIVED FOR PUBLICATION, SEPTEMBER 12, 1949)

In the usual bacteriological practice blood for blood cultures is taken from a vein. The incentive for utilizing capillary blood for cultures is based, in part, on a recent concept of the defence mechanism of the capillaries. According to this theory the capillaries filter off the circulating thrombi of bacterial cells and thrombocytes. It is a well-known fact that in subacute bacterial endocarditis the finger-tips are congested with thrombi. There is also reason to believe that in bacteriaemic conditions the concentration of bacteria is higher in the capillary than in the venous blood. Mention, indeed, is made in the literature (Stitt, Clough, and Clough, 1938) of positive typhoid cultures yielded by blood drawn from the finger, simple bile medium being used. In this paper methods for a wider application of the microtechnique are presented.

The cultivation of streptococci and pneumococci as well as of Gram-negative organisms from the capillary blood has been possible. Contamination is avoided by the selective bacteriostatic action of gentian violet as described by Fleming (1942) and Francis (1941) incorporated in the culture media.

Media

Three media are used.

Medium I.—Dehydrated " difco " brain-heart infusion with P.A.B. and agar, 38 g.; sodium citrate (tribasic), 2 g., dissolved and made up with distilled water to 1,000 ml. The prepared medium is distributed in test-tubes in amounts of 5–7 ml. and sterilized in an autoclave for 30 minutes at 15 lb. pressure. To each tube 0.05 ml. of sterile " bacto supplement A," containing the necessary amount of gentian violet, is added aseptically. For storage the tubes are wrapped in tinfoil or cellophane.

Medium II.—Kracke blood culture medium. The medium is distributed in quantities of 5–7 ml. in test-tubes and sterilized in an autoclave at 15 lb. pressure for 30 minutes. To each tube 0.1 ml. of " bacto supplement A " is added aseptically, and the test-tubes are wrapped as described above. Better results are obtained when the dehydrated medium is dissolved in fresh-water fish broth—e.g., using *Barbiceps longus* (Rappaport and Glikin, 1943) instead of water.

Medium III.—3% glucose bile medium. With this medium heavy precipitation after incubation indicates the presence of glucose fermenters (Rappaport, 1947).

Method

Blood is best drawn from the finger-tip or, in infants, from the heel. The site is disinfected by means of a pad of absorbent cellulose. The pads are made of rectangular pieces of absorbent cellulose (11 × 8 cm.) stapled to larger squares of parchment paper

with a margin all round of 1 cm. To sterilize, the pads are folded in the middle with the cellulose inside, placed in envelopes, and autoclaved. Before use the pad is removed from the envelope, opened, and the cellulose wetted with "septoform" (i.e., glycerin, 10 g., 40% formalin, 8 ml., sap. mol., 6 g., or spiritus saponatus, 30 ml., made up with alcohol to 1,000 ml., mixed and filtered). The pad is then wrapped round the finger-tip and kept in place by a rubber band. The disinfectant is allowed to act for three minutes, whereupon the finger is wiped dry with a second sterile pad. A superficial puncture is made with a lancet, flamed just before use, and a few drops of blood extracted. The blood is taken up either in a 1-ml. sterile syringe (the plunger lubricated with paraffin oil before sterilization) or in a small sterile pipette (Fig. 1). A rubber tube is connected to the upper end of the pipette, which is still held in the inner test-tube, and just before use the pipette is taken out of this tube. While blood is being drawn from the finger-tip, care must be taken that no air enters the pipette, as it may be a source of contamination. Each of the three media is aseptically inoculated with the blood and incubated from 12 to 16 hours for pneumococci, 24 to 48 hours for other organisms, and six to seven days in an atmosphere of 10% CO_2 for *Brucella*. One of the two smears made from the subcultures is stained by the Gram method and the other with methylene blue, both after alcohol fixation. Methylene blue is used because Gram-negative bacteria grown under these conditions take on the fuchsin counter-stain with difficulty. In methylene-blue-stained smears the micro-organisms of the enteric group appear as bipolar rods closely resembling *Pasteurella*.

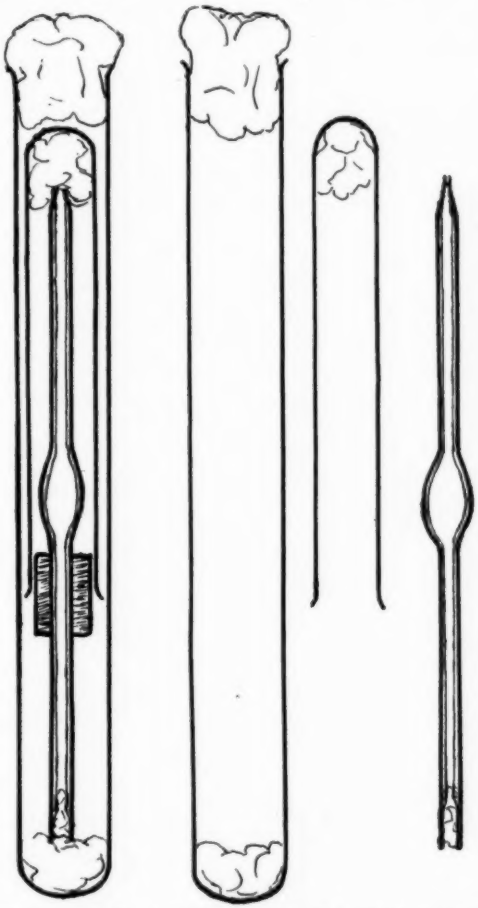

FIG. 1.—Sterile pipette for use in the microtechnique for blood cultures.

Discussion

The results obtained by this technique compared with those using venous blood have so far been satisfactory. Moreover, in some cases of subacute bacterial endocarditis in which venous blood cultures were negative the micro-method enabled streptococci to be cultured. Positive capillary-blood cultures, usually free from contaminants, were obtained in cases of typhoid fever

(*Salm. typhi*), paratyphoid (*Salm. para typhi* A, B, and C), subacute bacterial endocarditis (*Str. viridans*), scarlet fever (*Str. pyogenes*), pneumonia (*Str. pneumoniae*), and septicaemia (anaerobic streptococci). *Brucella melitensis* has also been isolated by this method.

In our experience one negative result should not be considered as final. If a septic condition is suspected most reliable results are obtained when cultures are repeated at daily intervals.

The technique we have described is much more convenient than venipuncture, particularly in cases where sufficiently prominent veins are difficult to find. The method is also of special importance when repeated blood culture from the same patient is required. At the very least, this method should prove to be a valuable auxiliary to the usual method.

References

Fleming, A. (1942). *Brit. med. J.*, **1**, 547.
Francis, A. E. (1941). *Lancet*, **2**, 159.
Rappaport, F. (1947). *Amer. J. clin. Path.*, **17**, 904.
—— and Glikin, T. (1943). *Acta med. orient., Tel-Aviv*, **2**, 148.
Stitt, E. R., Clough, P. W., and Clough, M. C. (1938). *Practical Bacteriology, Hematology, and Parasitology*. H. N. Lewis & Co.

J. clin. Path. (1950), 3, 77.

A TWO-SYRINGE METHOD FOR EXCHANGE BLOOD TRANSFUSION

BY

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(RECEIVED FOR PUBLICATION AUGUST 13, 1949)

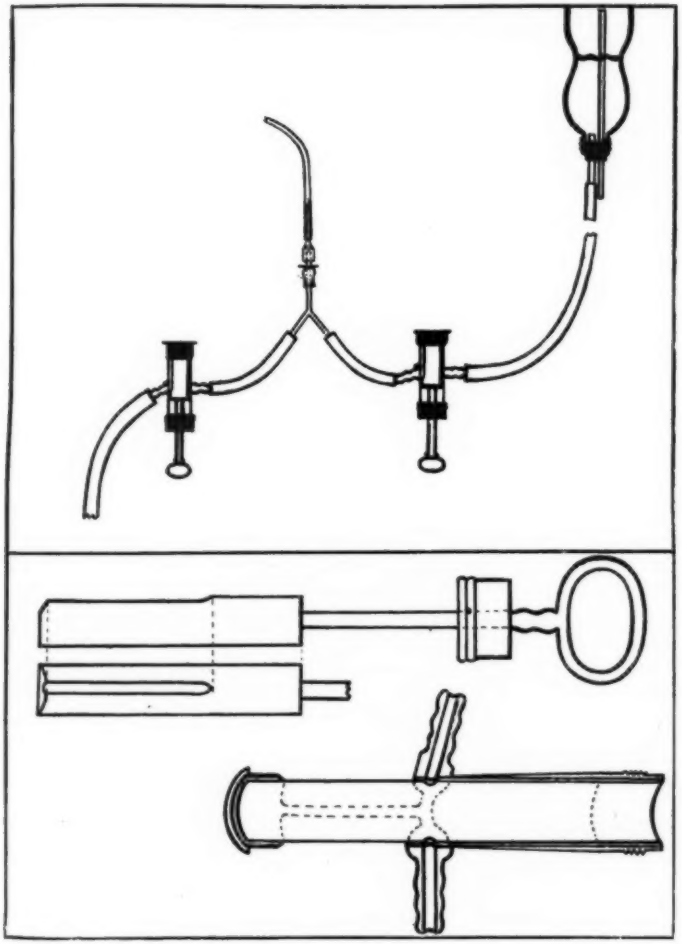
Exchange blood transfusion in cases of erythroblastosis foetalis is now an accepted form of treatment but a somewhat tedious one, especially if it has to be carried out in the early hours of the morning. It was, therefore, decided to try and see whether the time could be cut down by using two syringes, whereby blood was withdrawn from the infant into one while the other was being charged with blood from the bottle. The infant thus receives blood for every complete excursion of the piston intead of once in every two.

The illustration explains itself. A glass Y-piece with short rubber tubing and adaptor connects the needle from the umbilical catheter by two pieces of pressure tubing 5–6 in. long to the two syringes, one of which is connected to the bottle of blood and the other to the receptacle for the withdrawn blood. The syringes are of the Louis Jubé 5 ml. type, but any syringes with suitable two-way taps may be used. The great advantage of the Jubé syringe is that the alteration in the direction of flow is made by turning the piston through 180° and the hand does not have to be removed to turn a small tap. This is a time-saving measure.

A trial in the laboratory showed that with a little practice two people soon could work in unison, and the method has now been tried in a small number of cases with success. The time taken to transfuse about 450 ml. of blood is approximately 25 minutes.

Certain precautions are necessary. Both operators must work in unison: care is needed here, especially when the syringes are being emptied, because of the less resistance on the exsanguinating side. If an air bubble is seen in the glass Y-piece the transfusion must be stopped by both operators at once and the bubble removed. The rubber connexions between the Y-piece and the syringes must be long enough to prevent any pull on, and possible breakage of, the Y-piece (several spare Y-pieces are sterilized in case this happens, but so far they have not been needed). It is advisable to have a spare sterile syringe.

The advantage claimed for the method is the great saving of time. The disadvantage is that it requires two operators instead of one.



Summary

A method of carrying out exchange transfusion using two syringes is described. Certain precautions are noted.

[A possible criticism of this method is that the size of the syringe (5 ml.) is too small in relation to the dead space in the tubing and catheter. The exchange obtained with a small syringe is less effective ; in particular the advantage of the first withdrawal with a large syringe (wholly infant's blood) is lost. In practice, however, Dr. Barlow removes 25–30 ml. of infant's blood without transfusing and ends up with 35–40 ml. of blood without exsanguination.—ED.]

J. clin. Path. (1950), 3, 79.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Action of Chloromycetin on Salmonella. SELIGMANN, E., and WASSERMANN, M. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 253.

In an effort to find an effective remedy for salmonella infections in man and animals, the authors, working at the Beth Israel Hospital, New York, have examined the effect of chloromycetin (produced by *Streptomyces venezuelae*) on 68 Gram-negative strains *in vitro*. Of 23 *Salmonella* type 19, including *S. typhi*, were sensitive to 2 μg. per ml., and 4 to 4 μg./ml. No relation was found between streptomycin resistance and chloromycetin resistance. For experiments *in vivo*, chloromycetin was dissolved in 20% propylene glycol by heating for 10 to 20 minutes at 56° C., the final concentration being 10 mg. per ml. No toxic effects were observed when 2,000 μg. was given by mouth to mice twice daily for 4 days. In contrast to streptomycin, chloromycetin was absorbed from the intestinal tract and growth of the intestinal flora was not inhibited. In mice infected with inocula of 0.1 ml. of 18-hour broth cultures of *S. typhi-murium*, 2,000 μg. of chloromycetin by mouth twice daily for 4 days, or 2,500 μg. subcutaneously twice daily for 6 days and then once daily for 2 days, resulted in some prolongation of survival time. Parenteral treatment was superior to oral in two experiments. The action of chloromycetin was, however, bacteriostatic only and the drug had no curative effect. *Malcolm Woodbine.*

Typhoid Fever Treated with Aureomycin and Chloromycetin. (Su alcuni casi di tifo trattati con aureomicina e cloromicetina). SCARZELLA, M., MIGAZZO, C., and CAPPIO, M. (1949). *Minerva med., Torino*, **1**, 875.

The authors describe an outbreak of severe typhoid fever in Biella, Italy, in which 26 patients who had been treated unsuccessfully with typhoid vaccine and sulphonamides were given chloromycetin (8 cases) or aureomycin (18 cases). The patients' ages ranged from 7 to 54; 11 of them were male and 15 female. The dosage of aureomycin in adults was 125 mg. every hour for 3 hours, then 250 mg. every 2 hours until the fever subsided followed by 250 mg. every 4 hours for 48 to 72 hours.

Of the two drugs, chloromycetin gave the better results, the temperature falling within 3 to 5 days of starting treatment, headache disappearing, and the general condition improving, often in a most dramatic manner. Aureomycin was by no means as effective as chloromycetin; in one case the temperature dropped only after 10 days' treatment, while 4 patients did not improve at all, although they responded very well to chloromycetin given afterwards. In patients who responded to aureomycin the general condition improved more quickly than the fever subsided. No complications occurred during treatment with either drug, and where such complications existed before starting treatment they were quickly overcome. None of the 26 patients died.

In a control group of 62 patients the fever lasted 25 to 30 days, the mortality rate was 7%, and the incidence of complications 17%. The authors consider that the results obtained with these two drugs were excellent and beyond expectation.

Aureomycin in the Treatment of Penicillin-resistant Staphylococcic Bacteremia. NICHOLS, D. R., and NEEDHAM, G. M. (1949). *Proc. Mayo Clin.*, **24**, 309.

Of 50 strains of *Staphylococcus pyogenes* isolated at the beginning of 1949 in the clinical laboratory at the Mayo Clinic, 34 were resistant to penicillin. Of these 34 strains, 14 were resistant to streptomycin and all were sensitive to aureomycin. Details are given of the treatment with aureomycin of 6 cases of staphylococcal bacteriaemia due to penicillin-resistant strains, in which other forms of therapy had failed. Four patients recovered

Increase in Resistance of Tubercle Bacilli to Sodium *p*-Aminosalicylic Acid: Observations on Cultures Isolated from Patients During Chemotherapy. DELAUDE, A., KARLSON, A. G., CARR, D. T., FELDMAN, W. H., and PFUETZE, K. H. (1949). *Proc. Mayo Clin.*, **24**, 341.

Cultures of tubercle bacilli obtained from 73 patients were tested for *in vitro* resistance to sodium *p*-aminosalicylate (NaPAS). The patients were divided into 4 groups: (A) received no PAS (46); (B) received PAS (total 70 to 900 g.) for 94 days or less (18); (C) received PAS (total 850 to 1,684 g.), "promin," and streptomycin for 144 to 180 days (4); (D) received PAS (total 950 to 2,250 g.) for 157 to 251 days (5). Egg-yolk agar medium, to which solutions of NaPAS, sterilized by filtration, were added in graded concentrations, was used for resistance tests. The inoculum was 0.1 ml. of a ground suspension of the original culture at about 1 mg. per ml., and the highest concentration of the drug permitting growth after 30 days of incubation was taken as the end-point. The resistances of cultures isolated from groups (B), (C), and (D) before the start of PAS treatment were 0.006 to 0.012 mg. per 100 ml. of medium; as would be expected, the resistances in group (A) (no PAS treatment) were the same. At the end of treatment, the resistances of cultures from groups (B) and (C) had not increased, but those from 4 of the 5 patients in group (D) (receiving approximately the same amount of PAS as group (C)) were now resistant to 1.6 mg. to 6.4 mg. NaPAS per 100 ml. of medium, representing a 200- to 500-fold increase. The main difference between groups (C) and (D), in which the increases of resistance of cultures after treatment were so different, was the fact that PAS was combined with streptomycin and promin in the former group. [The development of strains of tubercle bacilli resistant to PAS by *in vitro* exposure to graded concentrations of the drug has not, so far as the abstracter is aware, yet been reported.]

P. D'Arcy Hart.

Roseola (Exanthem Subitum). HILL, L. F. (1949). *J. Iowa St. med. Soc.*, **39**, 283.

The author summarizes 38 cases, which show the classical features of an infection of sudden onset in children under the age of 3. The fever lasted for 3 to 5 days, without abnormal physical findings, then fell suddenly with the appearance of a morbilliform rash which lasted for 24 to 48 hours. The important laboratory finding was of a leucopenia and lymphocytosis. The cause was probably a virus of low infectivity, as the disease rarely spread from child to child. The differential diagnosis from measles and rubella is easily made from a study of the temperature chart and of the day on which the rash appears. In measles the exanthem appears at the height of the fever, in rubella at the start of the illness, and in roseola infantum as the temperature falls.

N. M. Jacoby.

BIOCHEMISTRY

Dextran as a Plasma Substitute. THORSEN, G. (1949). *Lancet*, **1**, 132.

Dextran is now produced commercially—"dextran Ph"—and is a polydispersoid glucose polymer whose average molecular weight is similar to that of albumin. It has been given to 5,000 patients in quantities up to 4 litres per infusion. It is non-toxic, non-antigenic, and virus free, and in Sweden to-day the hospitals rely on it largely for the prevention and treatment of all forms of shock.

After an infusion of 1 to 2 litres the dextran level in plasma rises to 1 to 2.5 g. per 100 ml. The fraction of molecular weight less than 25,000 is soon excreted in the urine, and the dextran level in the latter may rise to 7 g. per 100 ml. without any sign of renal damage. The fraction with higher molecular weight is presumably metabolized. Dextran slowly disappears from the circulating plasma. "In animals to which dextran has been given in repeated infusions, so that the total amount of dried substance corresponds to a third of the body weight, no dextran has been discovered in the brain, lungs, heart, muscle, liver, spleen, kidney or bone marrow . . . ." [Further evidence of this is highly desirable.]

The dextran exerts a colloid osmotic pressure and its infusion has corrected states of lowered plasma colloid osmotic pressure, notably in paralytic ileus with oedema of the intestinal wall. In the presence of dextran in the plasma, the plasma protein level may fall as low as 2 g. per 100 ml. without signs of disturbance of the mechanisms of coagulation of the blood or of the formation of antibodies. *John F. Loutit.*

Diagnostic Significance of Urinary Hormonal Assays: Report of Experience with Measurements of 17-Ketosteroids and Follicle Stimulating Hormone in the Urine. ESCAMILLA, R. F. (1949). *Ann. intern. Med.*, **30**, 249.

In this paper the results of a study of the daily urinary excretion of 17-ketosteroids and follicle stimulating hormone (FSH) in a number of endocrine and other disorders are recorded. The 17-ketosteroids have recently been subdivided into ketonic and non-ketonic fractions, of which the former has been further separated, by the action of digitonin, into alpha and beta fractions. It is suggested that further fractionation will enhance the diagnostic importance of the 17-ketosteroids, as certain of the compounds seem only to be present in particular clinical states.

High levels of excretion of the 17-ketosteroids were found in carcinoma or hyperplasia of the adrenal cortex, the excretion of the beta fraction being particularly increased in patients with carcinoma. Diminished excretion of the 17-ketosteroids was found in hypophysial infantilism, Simmonds's disease, anorexia nervosa, and Addison's disease. Daily variations in 17-ketosteroid excretion in normal subjects makes the finding of slightly high or slightly low values in a number of conditions of limited clinical value. FSH excretion was measured by bioassay and was found to be consistently high in ovarian aplasia (when the patient was not receiving oestrogen therapy) and occasionally at the male and female climacteric. A low level of FSH excretion was found in hypophysial infantilism, anorexia nervosa, and Simmonds's disease. A long list is given of disorders in which 17-ketosteroid and FSH excretion was normal.

Studies in Diabetic Acidosis and Coma, with Particular Emphasis on the Retention of Administered Potassium. DANOWSKI, T. S., PETERS, J. H., RATHBUN, J. C., QUASHNOCK, J. M., and GREENMAN, L. (1949). *J. clin. Invest.*, **28**, 1.

It is becoming well recognized that the hazards of recovery from diabetic coma include a risk of significant deficiency in serum potassium and total body potassium. This paper supplies a detailed quantitative description of the changes in electrolyte

and nitrogen metabolism in 7 patients who recovered from diabetic coma, one patient being observed on two separate occasions. The chief points brought out by the authors are as follows: (1) The serum potassium level was below normal on admission in about half the patients; even when this was not so it fell during the initial period of treatment of the coma with insulin, glucose, and isotonic saline. (2) When potassium chloride was given, either by mouth or intravenously, large amounts of potassium were retained; during a subsequent period without added potassium there was no excretion of the potassium retained in the previous period. The obvious conclusion is that a gross deficit of potassium had developed during coma—since potassium deficiency was present on admission—and also during the usual treatment, potassium balance being negative on the ordinary treatment. (3) As would be expected, large amounts of sodium, chloride, and carbohydrate were retained during recovery from coma. (4) Nitrogen was lost from the body during the early stage of observation and treatment, but was retained during recovery.

Discussing their results, the authors attribute the loss of potassium mainly to extracellular dehydration, with its transfer of water and potassium from the cells, and to the loss of potassium in the diuresis associated with diabetic coma. Subsidiary factors may be the loss of potassium normally associated with glycogen in the liver and with protein in the tissues, and the effect of interrupted carbohydrate metabolism in causing potassium to leave the cells.

HAEMATOLOGY

Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXVIII. Serum Iron Transport. Measurement of Iron-binding Capacity of Serum in Man. RATH, C. E., and FINCH, C. A. (1949). *J. clin. Invest.*, **28**, 79.

In cases of iron deficiency the serum iron level was found to be low, but the total iron-binding capacity was above normal; saturation was below 10%. In cases of chronic infection both the serum iron and total iron-binding capacity were reduced and the saturation was above 10%. The results in pregnancy and in other anaemias and the effects of injections of human globulin are also discussed.

Chemical, Clinical, and Immunological Studies on the Products of Human Plasma Fractionation. XXXIX. The Anemia of Infection. Studies on the Iron-binding Capacity of Serum. CARTWRIGHT, G. E., and WINTROBE, M. M. (1949). *J. clin. Invest.*, **28**, 86.

The total iron-binding capacity of serum is the sum of the serum iron content and the "unsaturated iron-binding capacity." The "percentage saturation" is calculated by dividing the observed serum iron content by the total iron-binding capacity. In patients with chronic infections the serum iron and total iron-binding capacity were found to be reduced and the percentage saturation lowered. The values rose in patients recovering from infections.

In two patients intravenous injection of metal-combining globulin raised the total iron-binding capacity to normal, but did not diminish the rate of disappearance of iron from the serum. Experimental work in dogs is also referred to.

Some Observations on the Effect of Folic Acid Antagonists on Acute Leukemia and Other Forms of Incurable Cancer. FARBER, S. (1949). *Blood*, **4**, 160.

In this important paper are summarized the results of the treatment of children with acute leukaemia by means of aminopterin and related compounds (amethopterin and amino-an-fol). Of 60 children, rather more than 50% showed temporary clinical

and haematological improvement. Two children were still alive 23 and 16 months after the onset of the disease.

The Use of Folic Acid Antagonists in the Treatment of Acute and Subacute Leukemia. A Preliminary Statement. DAMESHEK, W. (1949). *Blood*, **4**, 168.

The results are given of treatment with aminopterin and related compounds of 35 patients with acute or subacute leukaemia (31 adults). It is considered necessary to give doses large enough to cause general toxic effects. When remissions have occurred attempts have been made to continue treatment by smaller maintenance doses given orally. Amethopterin, amino-an-fol, and a-ninopterin are less toxic drugs, but less active therapeutically. A-ninopterin may, however, be slightly less toxic in effective doses than is aminopterin. Remissions occurred in nine of the 35 patients.

The Distribution Curve of Erythrocyte Fragility. A Different Method of Presentation of Fragility of Erythrocytes to Hypotonic Saline, with Preliminary Remarks on the Function of Reticulocytes. BOLTON, J. H. (1949). *Blood*, **4**, 172.

The author describes a method for recording erythrocyte fragilities (increment haemolysis curves) which demonstrate the distribution of fragility in much the same way as a Price-Jones curve gives the distribution of cell diameters amongst a cell population. Curves drawn in this way have demonstrated that the distribution of cell fragility may be bimodal or even trimodal in certain cases.

Haemolytic Disease of the Newborn: Criteria of Severity. MOLLISON, P. L., and CUTBUSH, M. (1949). *Brit. med. J.*, **1**, 123.

This paper contains much valuable factual information, and is based upon a study of 74 cases, mostly treated by exchange transfusion. Five infants were born dead and 19 died later. The haemoglobin level of the cord blood was found to be of prognostic significance; no infant with a value of 14 g.% or over died. Taken in conjunction with the haemoglobin level the bilirubin content of the cord plasma also afforded some indication of the severity of the disease. The strength of the direct Coombs reaction performed on the infant's corpuscles and the amount of free antibody in the infant's serum were, however, not found to be a reliable criteria of severity.

MORBID ANATOMY AND HISTOLOGY

The Effect of Total Thyroidectomy on the Function of Metastatic Thyroid Cancer. RAWSON, R. W., MARINELLI, L. D., SKANSE, B. N., TRUNNELL, J., and FLUHARTY, R. G. (1948). *J. clin. Endocrinol.*, **8**, 826.

Twenty-one patients with metastatic thyroid cancer were studied at the Massachusetts General Hospital, and the Memorial Hospital, New York. These patients had metastatic carcinomata which were considered relatively or absolutely non-functional because tracer experiments showed little or no ability to metabolize radio-iodine. In seven cases total thyroidectomy was carried out surgically: in the remainder the thyroid was destroyed by means of a large dose of radio-iodine (I^{131}). Tracer experiments were carried out before and after thyroidectomy to determine whether or not thyroidectomy altered the ability of the metastases to metabolize iodine.

Of these patients, 13 had solid and/or follicular adenocarcinomata, and eight of these metastatic tumours became able to concentrate radio-iodine after removal of the normal gland. This change occurred after periods varying between 1 month and 32 months. Four papillary adenocarcinomata failed to concentrate iodine after thyroidectomy. Three patients with giant-cell carcinoma died within six months after thyroid-

ectomy ; in these there was no change in the function of the metastases. In one case of metastatic Hürthle-cell adenocarcinoma no change in the function of the metastases has been observed up to the time of reporting.

Thyroid-stimulating hormone was given to one patient with a solid adenocarcinoma ; the metastases in this case did not collect radio-iodine after thyroidectomy and the patient became myxoedematous. After administration of thyroid-stimulating hormone, the metastases were found to collect significant amounts of radio-iodine.

[This paper is of great significance to those interested in the clinical application of radio-iodine and to oncologists in general. This is one of the few instances, if not the only instance, in which interference with normal physiology produces a definite and measurable alteration in the metabolism of neoplastic cells.] *Douglas Findlay.*

Pulmonary Adenomatosis. A Report of Three Cases. DRYMALSKI, G. W., THOMPSON, J. R., and SWEANY, H. C. (1948). *Amer. J. Path.*, **24**, 1083.

Pulmonary adenomatosis accounts for about 5% of all pulmonary tumours and probably results from non-specific irritation. Occurring in the sixth decade, and more commonly in women than in men, the disease runs a course varying from weeks to two years and is without distinctive clinical or radiological features, although it may simulate tuberculosis, carcinoma, or pneumonia. Macroscopically the lung resembles that of grey hepatization, while microscopically the alveolar walls are lined by uniform nonciliated cubical or columnar cells, which may assume papillary formations and which are probably of bronchiolar origin. Mitoses are infrequent. The disease is considered to be a benign variant of the so-called alveolar-cell carcinoma, and has many features in common with pulmonary adenomatosis in sheep (Jaagsiekte).

Of the cases reported here, one shows a small focus of malignant change in the adenomatous cells and another is the second surgical specimen of the condition to be recorded. *R. C. B. Pugh.*

Mast Cells. Their Distribution in Various Human Tissues. JANES, J., and MCDONALD, J. R. (1948). *Arch. Path.*, **45**, 622.

Mast cells were proved to be present in the subendothelial layer of synovial membranes, and their number to be increased in cases of chronic inflammation and tuberculosis. They were absent from cartilage, thyroid, kidneys, endometrium, umbilical cord, placenta, and scar tissue. They were more numerous in normal or acutely inflamed organs than in those affected by neoplastic growths. The authors regard it as likely that mast cells secrete heparin, and that this substance is the anticoagulant in synovial fluid. They indirectly confirm this assumption by recording that coagulation occurred in fluid from a haemarthrosis immediately after addition of protamine, a substance which is known to annul the action of heparin. *R. Salm.*

Congenital Pneumonitis in Newborn Infants. ADAMS, J. M. (1948). *Amer. J. Dis. Child.*, **75**, 544.

Six cases of primary virus pneumonitis are described. Characteristic shadows are seen in the radiographs of the lung. Pharyngeal smears contain large numbers of epithelial cells, and almost no bacteria or polymorphonuclear leucocytes. Cytoplasm inclusion bodies in the epithelial cells have diagnostic significance if present in large numbers. In some babies during an epidemic, sneezing and coughing occur without pneumonitis. The mortality rate is in direct relation to the severity of symptoms and signs. In all, pharyngeal smears from both the mother and the infant showed many inclusion bodies.

Ultra-violet Microscopy of Living Malignant Cells. LUDFORD, R. J., SMILES, J., and WELCH, F. V. (1948). *Nature, Lond.*, **162**, 650.

Cells of several kinds of transplantable malignant tumours, fibroblasts from subcutaneous connective tissues, and mesothelial cells were studied. To avoid unnecessary exposure to ultra-violet irradiation suitable preparations were selected by dark-ground illumination. Preliminary studies of the cells were made by phase-contrast microscopy in order to detect signs of injury to cells exposed to ultra-violet irradiation. With objectives computed to wavelengths of 2,570 Å and 2,750 Å, photomicrographs of living cells were obtained without causing appreciable injury to cell structure. It was assumed, since living cells were irreversibly injured by a series of exposures to irradiations of varying wavelengths, that structures showing strong absorption with wavelengths of 2,570 Å and 2,750 Å contained nucleotides.

Mitochondria exposed to ultra-violet irradiation become distorted, then disintegrated, and finally replaced by vacuoles. The cytoplasm of rapidly growing malignant cells contains much absorbing substance. During cell division a regular sequence of changes in the distribution of nucleotides was observed. When the nuclear membrane breaks down at prophase the previously filamentous mitochondria become rounded and carry peripheral deposits of nucleotides. "Ground cytoplasm" is almost devoid of absorbing substance. Chromosomes show strong absorption throughout mitosis. The mitochondria resume their filamentous shape during the last stages of cell division, parallel with the reappearance of nucleotides in the cytoplasm. At this time the chromosomes are clearly distinguishable in the reconstituting nuclei, and nucleoli have not reformed. The nucleus is often surrounded by a relatively clear zone, and no indications were found of a higher concentration of absorbing material immediately bordering the nuclear membrane. Mitochondria rather than nucleoli appear to be implicated in the synthesis of cytoplasmic nucleoprotein. Under suitable conditions nuclei can be seen to contain fine double threads bearing parallel pairs of minute granules which are presumably chromosomes or possibly gene loci. A definite correlation exists between the amount of nucleotides and the rate of cell growth. *A. K. Powell.*

Carcinoma Developing in Sebaceous Cysts. PEDEN, J. C. (1948). *Ann. Surg.*, **128**, 1136.

Evidence of carcinoma was present in 14 of 832 sebaceous cysts subjected to microscopy—an incidence of 1.7%. Of the 14, 7 were removed from the scalp, 2 from the face, and one each from the ear, shoulder, arm, forearm, and thigh. Though the incidence does not justify the view that sebaceous cysts are pre-cancerous, it points to the need for their routine histological examination after removal. The growth is usually a highly differentiated squamous-cell carcinoma of low malignancy, and the lymph nodes are not commonly involved. Suspicion of the complication may be aroused if in older people an increase in the size of the cyst has been noted, or pain develops, and under such circumstances treatment should be by wide local excision.

Harold C. Edwards.

A Reorientation on the Histogenesis and Clinical Significance of Cutaneous Nevi and Melanomas. ALLEN, A. C. (1949). *Cancer*, **2**, 28.

This is a careful and well-illustrated study of the histogenesis of pigmented moles and melanomata. The author concludes that these arise, not from any specialized neural cells, but from the ordinary cells of the epidermis. He distinguishes between "junctional naevi," in which the naevus cells are still within or connected with the epidermis, and intradermal naevi, in which the naevus cells have migrated from the epidermis; the former is the usual type in children, the latter in adults. Transitional or combined naevi are frequent. The blue naevus and Mongolian spot, however, are not epidermal in origin but probably neurogenic. *R. A. Willis.*

J. clin. Path. (1950), 3, 86.

BRANCH REPORTS

The autumn meeting of the **Channel and S.E.** branch was held at the Royal East Sussex Hospital, Hastings, on October 8, 1949.

The programme included the following papers and demonstrations:—

Papers: J. McMurray, "The Value of Biopsy Examinations"; P. Lazarus-Barlow, "Three Interesting Cases"; M. Haines, "The Male Toad Test for Pregnancy"; C. A. Holman, "Spontaneous Remission in a Case of Myeloid Leukaemia"; M. O. Skelton, "Two Malignant Ulcers in a Stomach"; M. Sadiq, M. O. Skelton, and H. G. Close, "A Case of Hepatoblastoma."

Demonstrations: A. G. Shera, "Secondary Bronchogenic Carcinoma Simulating Femoral Hernia"; Myra K. Beatty, "Multicystic Ovaries with Characters of Arrhenoblastomata"; H. G. Close, "Myelo-lipoma of the Suprarenal"; P. Lazarus-Barlow, "Two-syringe Method for Exchange Transfusion" (see p.—); "? Primary Carcinoma of the Liver"; "Carcinoma of Bronchial Cyst"; "Primary Carcinoma of Fallopian Tube"; "Ruptured Heart Muscle due to Passage of Electric Current"; J. McMurray, "? Endometriosis in Small Intestine causing Fibrosis and Obstruction"; "Krohn's Disease"; "Hydronephrosis due to Aberrant Vessels and Pelvic Stone"; "Aneurysm of Circle of Willis"; "Oesophagus and Stomach from a Case of 'Izal' Poisoning"; "Mid-brain Tuberculoma as Precipitating Focus of Meningitis"; "Intestinal Infarct"; "? Lymphosarcoma of Cervical Glands Diagnosed as Diphtheria"; "Healed Endocarditis showing Aneurysmal Sacs on Valve Cusps"; "Contusion of Ventricles of Heart"; "Cystic Lung in a Case of Cerebral Abscess"; "Aneurysm of the Aorta in a Male of Twenty-four"; "Colon in a Case of Post-*x*-ray Colitis"; C. J. Harwood-Little, "Large Solitary Plasmacytoma"; F. Standish, "An Unusual Specimen."

A meeting of the **West Mercian** branch was held at Dudley Road Hospital, Birmingham, on October 15, 1949, when the following papers were read and specimens demonstrated.

Papers: S. Sevitt, "An Investigation into Two Hospital-infected Cases of Tetanus"; P. Kidd, "The Chown Method of Rhesus Typing: a Warning."

Demonstrations: H. S. Barr, "Congenital Granular Cell Myoblastoma of the Gum"; "A Unique Form of Intestinal Obliteration"; "Accessory Tracheal Bronchus without Accessory Lung"; B. T. Davis, "Eosinophil Leukaemia"; G. H. Grant, "Myxoma of Bladder"; F. E. D. Griffiths, "Two Cases of Renal Abnormalities"; P. Kidd, "? Reticulosis of Stomach"; "Two Tumours from the Fingers for Diagnosis"; Ian Mackenzie, "Pulmonary Adenomatosis"; A. G. Marshall, "A Case of Bone Marrow Dysplasia for Diagnosis"; A. P. Prior, "Series of Chorio Allantoic Membranes"; C. W. Taylor, "Pulmonary Embolism by Amniotic Fluid"; K. S. Thompson and G. Cruikshank, "Phase Contrast Microscopy for Urine and Body Fluids"; K. S. Thompson and G. Gilliatt, "Uterine Endometriosis"; W. Whitelaw and A. E. Chaplin, "Bronchial Adenoma"; "Tumours of the Thyroid."

J. clin. Path. (1950), 3, 87.

TECHNIQUES FOR THE EVALUATION OF ADRENAL CORTICAL FUNCTION BY THE USE OF ADRENOCORTICOTROPHIN: A REVIEW

BY

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The number of conditions in which abnormal adrenal cortical function can occur is an ever-widening field, including certain well recognized endocrine abnormalities in addition to extremes of environment, trauma, "non-specific" disease processes, and surgical procedures which may excite the so-called "stress reaction" (Selye, 1946). In addition, alteration in adrenal cortical activity seems to occur in some psychotic states. A short classification of conditions in which adrenal cortical function varies from normal is as follows:

Increased Adrenal Cortical Activity	**Decreased Adrenal Cortical Activity**
Cushing's syndrome	*Acute Insufficiency*
Adrenogenital syndrome	Waterhouse–Friderichsen syndrome
Oestrogen-producing tumours of the adrenal cortex	Following adrenal operations
Active stages of acromegaly	*Chronic Insufficiency*
Pharmacological response to adrenocorticotrophin therapy	Addison's disease
"Resistance phase" of stress reaction	Panhypopituitarism
	Myxoedema
	"Exhaustion stage" of stress reactions
	Schizophrenia
	(Decreased adrenal cortical "functional reserve." Pincus, Hoagland, Freeman, and Elmedjian, 1949).

It is becoming of considerable importance to possess rapid and easy criteria for detecting variations of adrenal cortical activity. Before considering relevant laboratory, as opposed to clinical procedures, for detecting abnormalities of function, the present concept of the physiological relations of the adrenal cortex in the organism must be outlined.

Physiological Relations of the Adrenal Cortex

That the adrenal cortex is immediately under the control of the anterior pituitary, which secretes adrenocorticotrophin (ACTH) is indicated in Fig. 1. Since the early

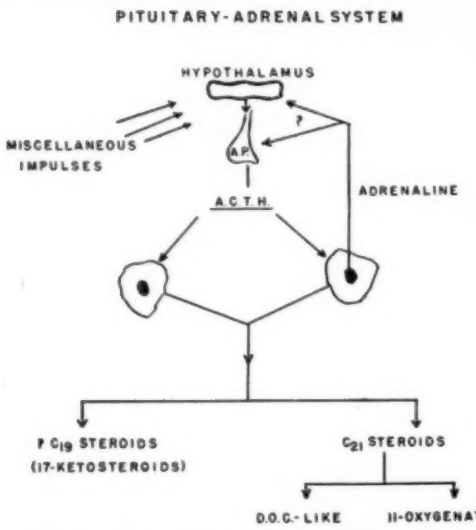


FIG. 1.—Physiological relations of the adrenal cortex.

work of Evans (1923–4) and Smith (1930) showing that the anterior pituitary is required for adrenal cortical maintenance in animals, great strides have been made in the separation and preparation of a pure protein possessing the activity of ACTH (Collip, Anderson, and Thomson, 1933; Sayers, White, and Long, 1943; Li, Evans, and Simpson, 1943). This hormone has now been shown to be obtainable as an active peptide (Li, 1950; Morris and Morris, 1950). In man the purified protein ACTH has been found to stimulate all the known metabolic activities of the adrenal cortex (Forsham, Thorn, Prunty, and Hills, 1948; Mason, Power, Rynearson, Ciaramelli, Li, and Evans, 1948), and since that time rapid advances have been made in the study of this substance. The activity of ACTH is mediated through the secretion of cortical steroid substances which are probably synthesized from the cholesterol contained in the gland (Long, 1947). The exact nature of the steroids secreted by the cortex is not known with certainty. Metabolic and clinical data show that activity which is known to be obtained with 11-oxygenated and 11-desoxy C_{21} steroids is manifest on stimulation of the adrenal cortex (Fig. 2). There is also evidence from the increased excretion of 17-ketosteroids to show that the C_{19} steroids are produced as a result of this stimulation, and previously urinary 17-ketosteroids were known to be derived from the adrenal cortex (Callow, 1938; Callow, Callow, and Emmens, 1938). The isolation of steroids from the adrenal suggests that 17-hydroxy-11-dehydrocorticosterone ("cortisone"; "Compound E" of Kendall) and 17-hydroxy-corticosterone ("Compound F" of Kendall) are synthesized by the gland (Reichstein and Shoppee, 1945; Haines, Johnson, Brunner, Pabst, and Kuizenga, 1949). These compounds are representative of 11-oxygenated C_{21} steroids, and they may also appear in the urine; for instance, large amounts of "Compound F" have been isolated from the urine of a patient with Cushing's syndrome by Mason and Sprague (1948). On the other hand very small quantities of desoxycorticosterone and C_{19} steroids only have been isolated from adrenals, and doubts exist that they may be artefacts of the process; it is therefore uncertain that the adrenal in fact synthesizes these compounds. Studies of the chemistry of 17-ketosteroids isolated from the urine suggest an origin of some at least of these excretory products as C_{21} adrenal steroids, which may be metabolized in the body at some site remote from the adrenal cortex, possibly the liver (Lieberman, Dobriner, Hill, Fieser, and Rhoads, 1948).

It will be seen that the precise nature of the steroids secreted by the gland is not at present clear, nor is the endogenous metabolism of these substances nearly fully elucidated. Fig. 1 illustrates the possibility of stimulating the anterior pituitary

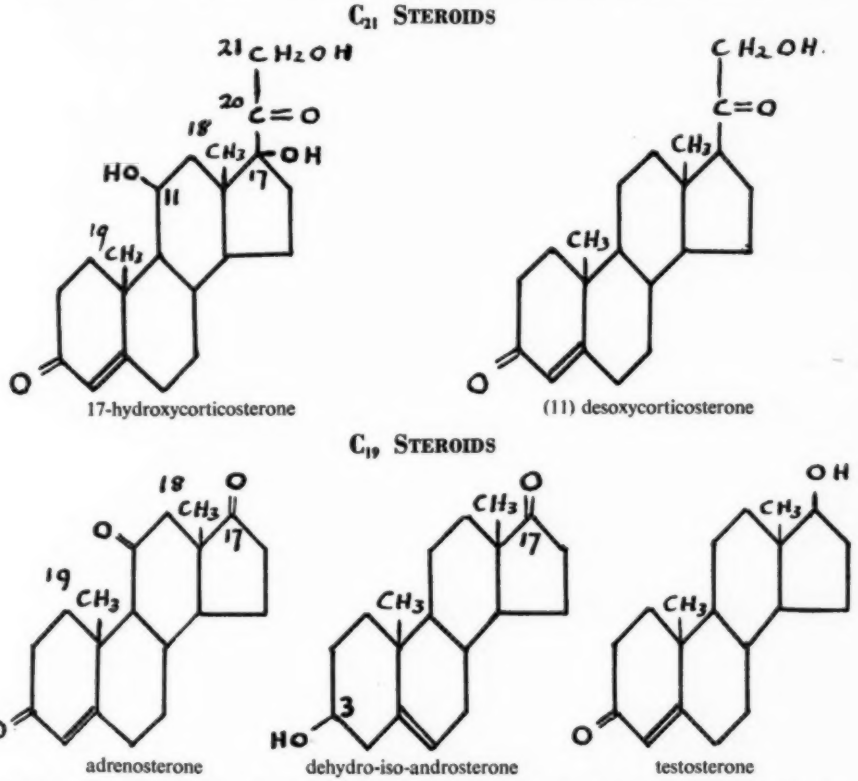


FIG. 2.—Formulae of some steroid compounds. Adrenosterone and dehydro-iso-androsterone are 17-ketosteroids; testosterone, for comparison, is a C_{19} steroid but not a 17-ketosteroid.

to secrete ACTH by means of adrenaline. The experiments of Vogt (1945) with prolonged adrenaline injections in normal and hypophysectomized animals, and of Long (1947) using short-term experiments, would seem to indicate that adrenaline stimulates the anterior pituitary to secrete ACTH. There is an admitted difficulty in that an earlier experiment of Vogt (1944) showed that adrenaline could directly stimulate the cortex in a decapitated dog. Recent work by Spiers and Meyer (1949) using eosinophil counts in mice confirms that the main action of adrenaline is on the anterior pituitary, but there may be some direct action on the cortex. This matter is further complicated by the report of Hume (1949) showing that damage to certain parts of the hypothalamus results in failure of the pituitary-adrenal

system to respond to adrenaline, and suggesting that the latter acts directly on the former structure. Nevertheless the presence of an intact pituitary is necessary for the normal reaction of the organism to stress stimuli (Long, 1947). It seems then that it may be taken as a working hypothesis that adrenaline is capable of causing ACTH production by the anterior pituitary which results in adrenal cortical stimulation. Adrenal steroids are effective in acting as a break on ACTH secretion by the pituitary (Sayers and Sayers, 1949).

Indications of Disturbance in Adrenal Cortical Function

Before the use of ACTH for evaluating adrenal cortical function, reliance had to be placed on the use of procedures summarized in Table I. Included in Table I

TABLE I

METHODS OF ASSESSING ADRENAL CORTICAL FUNCTION

	Hypofunction (Addison's Disease)	Hyperfunction (Cushing's Syndrome)
A. C_{21} STEROIDAL ACTIVITY		
1. 11-*oxygenated steroids:*		
(i) Carbohydrate metabolism		
Fasting blood sugar	Low	High
Intravenous glucose curve ..	Hypoglycaemic phase	Diabetic-like
Insulin sensitivity	Great	Small
(ii) Tissue response		
Lymphoid (circulating lymphocytes)	High	Low
Eosinophils	May be high	,,
(iii) Steroid excretion		
Neutral reducing steroids ..	Low	High
2. *Salt-retaining steroids:*		
Electrolyte metabolism		
Haematocrit	High	—
Serum sodium	Low	High
Serum chloride	,,	Low
Serum potassium	High	,,
Serum carbon dioxide	Low	High
Cutler Low NaCl, high K regime:		
Urine Na	High	—
Cl	,,	—
Administration of desoxycorticosterone acetate	Chloride retention	Chloride loss
3. Kepler diuresis test	Impaired	—
B. C_{19} STEROIDS		
17-ketosteroid excretion	Low	Normal or high
3 β-hydroxy 17-ketosteroids	—	Sometimes high in tumours

is the use of eosinophil counts, the value of which did not, however, become apparent until later. The tests in this table are classified under the headings of the types of steroid, the function of which they may be considered to be an index. In many respects the table is self-explanatory, but a few comments may be needed. That important changes occur in the blood counts of patients with adrenal cortical abnormality was pointed out by de la Balze, Reifenstein, and Albright (1946) as a result of experiments in animals at that time. Patients with Addison's disease have exces-

sive amounts of lymphoid tissue accompanied by high blood lymphocyte counts, and the reverse is true in Cushing's syndrome. In 1948 the importance of the eosinophil in relation to the adrenal cortex became apparent (Hills, Forsham, and Finch; Forsham *et al.*), and in Cushing's syndrome eosinopenia may be profound (Thorn and Forsham, 1949). In Addison's disease there may be a high eosinophil count, but statistically this is not significant and suggests that the control of the basal level of circulating eosinophils is governed by factors other than the adrenal cortex.

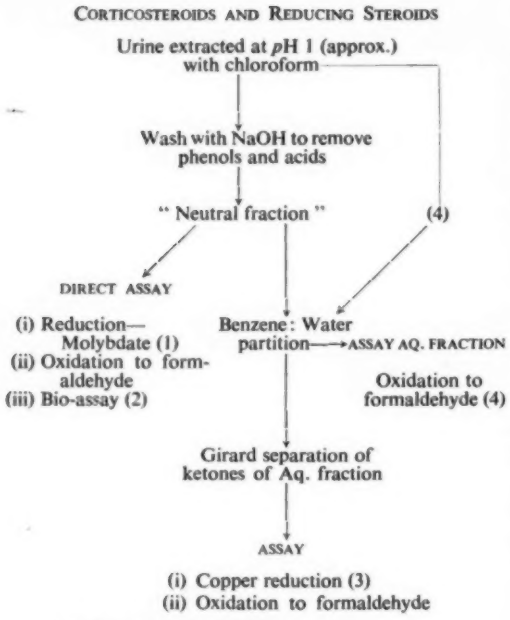
The rationale of the test described by Robinson, Power, and Kepler (1941), in which diuresis after the injection of water is impaired in Addison's disease, is more difficult to interpret. It is not restored to normal by the control of the patient with desoxycorticosterone, and there is some evidence that this alteration of adrenal function may be dependent upon deficiency of 11-oxygenated steroids (Gaunt, 1946). Delayed diuresis may occur in diseases other than Addison's disease where there is deficiency of water absorption, abnormal hydration, or renal insufficiency (Levy, Power, and Kepler, 1946). The specificity of the test is considerably enhanced by applying the second part described by the authors in which additional account is taken of the frequently associated decrease in urea clearance and increase of clearance of chloride. If this is done about 12% of cases give false negatives, and in practice it proves to be a most useful screening procedure.

Alterations in blood electrolyte composition cannot be regarded as very sensitive indices of changes of activity dependent upon salt-retaining steroids. In Addison's disease, even in instances where plasma volume is decreased, the normal concentration of electrolyte tends to be maintained until late, in spite of a total body deficiency of sodium. By the application of the test devised by Cutler, Power, and Wilder (1938) this system may be put under stress. The test represents a more carefully controlled procedure employing the older methods of applying stress to the adrenal by salt withdrawal. The patient is given a standard low salt diet with increased potassium intake for two days, and the excretion of sodium and chloride is estimated in the urine. In adrenal cortical failure the amounts of these remain high compared with the falling values in patients who do not have Addison's disease. In the absence of renal disease the test has a high degree of specificity (Willson, Robinson, Power, and Wilder, 1942), but suffers from the grave disadvantage that the patient with Addison's disease is subjected to considerable danger from the risk of an adrenal crisis, and for this reason it is becoming superseded by more recent techniques.

Excess secretion of salt-retaining steroids in Cushing's syndrome might be supposed to occur. The response of such patients to administration of desoxycorticosterone acetate has in fact been observed to be the reverse of that found in Addison's disease in 10 of 15 patients with increased adrenal cortical function (Soffer, Gabrilove, and Jacobs, 1949). The response to these steroids must therefore depend upon the existing salt balance in the body and on the magnitude of the activity of the adrenal cortex at the time of the observation (Prunty, 1949a).

"Neutral Reducing Steroids" and "Glyco-corticoids"

The estimation of the urinary excretion of "reducing steroids" has come to be of considerable value in assessing adrenal cortical function, in spite of the lengthiness of


CORTICOSTEROIDS AND REDUCING STEROIDS
Urine extracted at pH 1 (approx.) with chloroform
Wash with NaOH to remove phenols and acids
" Neutral fraction "
(4)
DIRECT ASSAY
(i) Reduction—Molybdate (1)
(ii) Oxidation to formaldehyde
(iii) Bio-assay (2)
Benzene : Water partition
ASSAY AQ. FRACTION
Oxidation to formaldehyde (4)
Girard separation of ketones of Aq. fraction
ASSAY
(i) Copper reduction (3)
(ii) Oxidation to formaldehyde

(1) Heard and Sobel (1946).
(2) Venning, Kazmin, and Bell (1946).
(3) Talbot, Saltzman, Wixom, and Wolfe (1945)
(4) Daughady, Jaffe, and Williams (1948a).

FIG. 3.—Principles of the estimations of reducing steroids and glyco-corticoids.

the procedures. The principles of this estimation are shown schematically in Fig. 3, where it will be seen that a variety of methods are at present available. It is not yet known which of these techniques is the most valuable as an indication of cortical steroid excretion, but there is in disease a general correlation between them and the biological technique of Venning, Kazmin, and Bell (1946), based on the ability of the urine extracts to cause deposition of glycogen in the mouse liver. The test measures the glyco-corticoids which may be assumed to be a biological index of excretion of steroids of the 11-oxygenated class. This estimation of the glyco-corticoids is a somewhat laborious technique, not suited to general use. The application of the chemical methods is, therefore, more widely favoured, and technically probably the easiest is the molybdate method of Heard and Sobel (1946) for reducing lipids. The use of the benzene : water partition step in the fractionation of the crude extracts and Girard separation favours the concentration in the extract of ketones with hydroxyl groups at the 11,17 positions, for example " Compound F." It is nevertheless questionable whether these steps are entirely desirable in a test

TABLE II

CORTICAL STEROID EXCRETION BY DIFFERENT TECHNIQUES

	Sex	Range	Average
Venning and Browne (1949)	Females	25–65 units	41 units
	Males	40–85 units	60 units
Heard, Sobel, and Venning (1946) ..	Females	1.0–2.0 mg./day	1.3 mg./day
	Males	1.1–2.1 mg./day	1.5 mg./day
Talbot, Albright, Saltzman, Zygmuntowicz, and Wixom (1947)		0.1–0.44 mg./day	0.22 mg./day
Daughady *et al.* (1948b)		1.0–1.6 mg./day	—

designed to give a general indication of cortical steroid excretion. The normal adult values by these methods are summarized in Table II.

There is general agreement among the devisers of these methods that in Cushing's syndrome, in active stages of acromegaly, and in conditions of stress, a marked rise above the normal may be expected, whereas in Addison's disease and hypopituitarism low values are to be expected, particularly in the latter. The fact that the levels rarely reach zero in these conditions means that some of these substances are of extra-adrenal origin.

Urinary Excretion of 17-ketosteroids

The general processes involved in this estimation are shown schematically in Fig. 4. For routine use estimation of the total 17-ketosteroid in the neutral fraction by the Callow *et al.* (1938) method is most applicable, but in applying the test in patients with low values correction for non-ketosteroid chromogens by the colour method of Fraser, Forbes, Albright, Sulkowitch, and Reifenstein (1941) is necessary to avoid falsely high results. Decreases of 17-ketosteroid excretion may be observed in a wide range of debilitating diseases and occasionally under conditions of stress—e.g., post-operatively (Venning and Browne, 1949). Values approaching zero are to be expected in patients with widespread damage to the anterior pituitary, whilst levels below normal are obligatory in the diagnosis of Addison's disease; in 37 male patients with the latter condition the range of 17-ketosteroid excretion was 1.0–8.0 mg. per day with a mean of 4.2, mean deviation ±2.7, and 33 female patients 0.1–8.6 with a mean of 3.0 and mean deviation ±2.0 (Thorn, Forsham, Prunty, Bergner, and Hills, 1949). In Cushing's syndrome related to adrenal cortical tumour the 17-ketosteroid excretion is frequently increased (Kepler, Sprague,

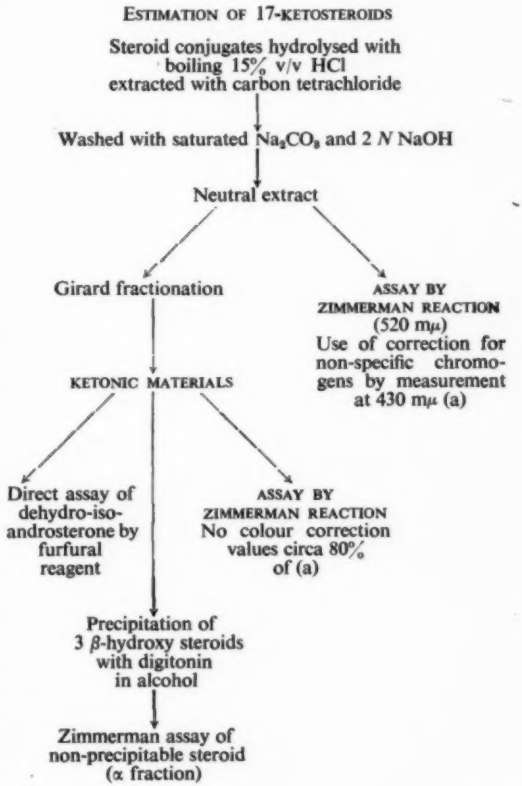

ESTIMATION OF 17-KETOSTEROIDS
Steroid conjugates hydrolysed with boiling 15% v/v HCl extracted with carbon tetrachloride
Washed with saturated Na_2CO_3 and 2 *N* NaOH
Neutral extract
Girard fractionation
ASSAY BY ZIMMERMAN REACTION (520 mμ) Use of correction for non-specific chromogens by measurement at 430 mμ (a)
KETONIC MATERIALS
Direct assay of dehydro-iso-androsterone by furfural reagent
ASSAY BY ZIMMERMAN REACTION No colour correction values circa 80% of (a)
Precipitation of 3 β-hydroxy steroids with digitonin in alcohol
Zimmerman assay of non-precipitable steroid (α fraction)

FIG. 4.—Principles of the extraction and fractionation of 17-ketosteroids.

Mason, and Power, 1948), and may be helpful in differentiating this from the syndrome associated with adrenal hyperplasia in which 17-ketosteroid levels are not greatly raised. High values for 17-ketosteroid excretion are frequently found in patients with the adrenogenital syndrome, and a value above 100 mg. per day is likely to be indicative of the presence of tumour rather than adrenal hyperplasia (Warren, 1945 ; Callow and Crooke, 1944), both of which conditions may occur in this syndrome. On the basis of the isolation of large amounts of dehydro-iso-androsterone, a 3 β-hydroxy-17-ketosteroid, in cases of tumour (Crooke and Callow, 1939 ; Mason, 1948) it has been suggested that this estimation is of value in the diagnosis of adrenal cortical tumour, values of not less than 50% of the total 17-ketosteroid being diagnostic of this lesion (Talbot, Butler, and MacLachlan, 1940a ; Callow and Crooke, 1944). The estimation of steroids of this type (Fig. 2) may conveniently be performed in two ways, by the precipitation of these compounds with digitonin and estimation of the 3 β-steroids in solution by the Callow method (Talbot *et al.*, 1940b), or by direct estimation in the ketonic fraction (Fig. 4) with the use of the furfural reagent (Munson, Jones, McCall, and Gallagher, 1948). That good agreement can be obtained by these methods is shown by the results in Table III (Prunty, unpublished).

TABLE III

ESTIMATION OF STEROIDS

Patient			Diagnosis	Total 17-ketosteroid (mg./day)	β-17-keto-steroid (% of Total)	Dehydro-iso-androsterone (% of Total 17-ketosteroid)
No.	Sex	Age				
1	F	40	Adrenal cortical carcinoma	178	34	36
2	F	21	,, ,, ,,	29.8	4	—
3	M	15	Adrenal cortical hyperplasia	57.5	29	31
4	F	18	,, ,, ,,	35	39	—
5	F	5	,, ,, ,,	34	15	18
6	M	3	,, ,, ,,	24.4	19	21
7	F	15	,, ,, ,,	108	26	23
8	F	15	,, ,, ,,	35.5	30	23
9	M	7	,, ,, ,,	20.3	19	—
10	M	2½	Interstitial cell tumour of testes	19.6	3	13.6
11	M	5½	,, ,, ,,	2.9	—	—
12	M	45	Cushing's syndrome	18.3	8	9
13	M	26	,, ,,	32.5	12	12

The estimation of the 3 β-hydroxy-17-ketosteroids might prove to be of value in the differentiation of true interstitial cell tumours of the testes from those arising from " adrenal rests," for testosterone is not metabolized to dehydro-iso-androsterone (Dorfman, 1948) and has not been isolated in the case of interstitial cell tumour described by Hoffman (1944). It is interesting to compare these results with Case 10, Table III. In our experience (Table III, Prunty, unpublished data), and of others, adrenal cortical tumour is not necessarily accompanied by very high values for 3 β-hydroxy-17-ketosteroids, whereas moderate elevations may occur in hyperplastic lesions. It would seem to be unexpected that patients with tumour excreting only moderate levels of total 17-ketosteroid, would show elevation of 3 β-17-

ketosteroid excretion (see Case 2, and Kepler and Mason, 1947). Dehydro-iso-androsterone was readily isolated with the aid of chromatography from the urine of Case 1. Table III also shows that although large amounts of 17-ketosteroid may be excreted in cases of interstitial cell tumour of the testes (Hoffman, 1944) this need not necessarily be so (compare Cases 10 and 11). In interpreting elevated levels of 17-ketosteroid excretion it should not be overlooked that trauma may be the cause of an increase, but this is not necessarily correlated with the values for neutral reducing steroids (Venning and Browne, 1949).

The Response of the Adrenal Cortex to ACTH

While for the most part the procedures in Table I test the function of the adrenal cortex in the resting state in the patient, it is now possible to stimulate the adrenal directly and so determine the functional reserve of the organ and the capacity to increase its activity. From the work of Long (1947) and of Sayers and Sayers (1949), it is apparent that the adrenal cortex reacts by the sudden release of available steroid hormones when a brief stimulus is applied, whereas with prolonged stimulation, in the absence of adrenal damage, there ensues hypertrophy of the gland with increasing steroid production. These principles may be applied in the use of ACTH in clinical work. In 1946 this material became available in the U.S.A. for clinical study in sufficient quantity to make possible extended investigations into its properties.* Two types of procedure were employed by Forsham, Thorn, Prunty, and Hills (1948): first the effects of the administration of a single dosage of ACTH, 25 mg. being that arbitrarily selected, and secondly the effects of more prolonged injection. From animal work it was apparent that ACTH must be administered in divided doses as frequently as possible, and it is now known that the life of the hormone in the body is very brief (Sayers, Burns, Tyler, Jager, Schwartz, Smith, Samuels, and Davenport, 1949; Li, 1950). It will be convenient first to consider the effects of prolonged administration of ACTH, and these effects may be classified under the headings of the types of steroid by which they are produced.

Effects of Prolonged Administration of ACTH

Stimulation of Activity Due to 11-oxygenated Steroids.—Evidence of increased activity due to the augmented secretion of these steroids may conveniently be obtained by measurement of changes in excretion of reducing steroids, alterations in carbohydrate metabolism, increased nitrogen excretion, uric acid metabolism, and changes in blood cell counts.

Increased Excretion of "Reducing Steroids."—It has been consistently found that ACTH produces an elevation in the excretion of "neutral reducing steroids." Daily doses of 40 mg. per day divided into four doses over a period of four days produced as much as a fivefold increase in the excretion of these steroids in a normal male, in a male with mild pituitary deficiency, and in a girl with asthenia due to a neurosis (Fig. 5). Doses of 240 mg. and more have been found to reproduce an increased excretion of glycocorticoids (Venning, Kazmin, Ripstein, McAlpine, and Hoffman, 1948), in as brief a period as 12 hours. There is a rapid return to normal levels after withdrawal of ACTH.

* By courtesy of Dr. J. R. Mote, of the Armour Laboratories, Chicago.

Alterations in Carbohydrate Metabolism.—Doses of 40 mg. ACTH daily produce small but significant rises in the fasting blood sugar level, from approximately 80 to 120 mg. %. In the experiments of Forsham *et al.* (1948) this tended to be maximal in the first 24 hours after the beginning of ACTH administration and then gradually to decline. No glycosuria occurred in these experiments, but with doses of 52 mg./day Conn, Louis, and Johnston (1949) obtained truly diabetic-like changes in blood glucose levels and glucose tolerance, with pronounced glycosuria, and the latter is being widely observed (Elkinton, Hunt, Godfrey, McCrory, Rogerson, and Stokes, 1949). It was possible to observe in one of our patients changes in liver glycogen following 48 hours' treatment with 80 mg. ACTH, an increase of 2.2 g. % (wet weight) being produced. Such a change is characteristic of the action of 11-oxygenated steroids (Reinecke and Kendall, 1943). Diabetic patients are liable to show much greater elevations of fasting blood sugar than are normal individuals during the use of ACTH (Thorn and Forsham, 1949).

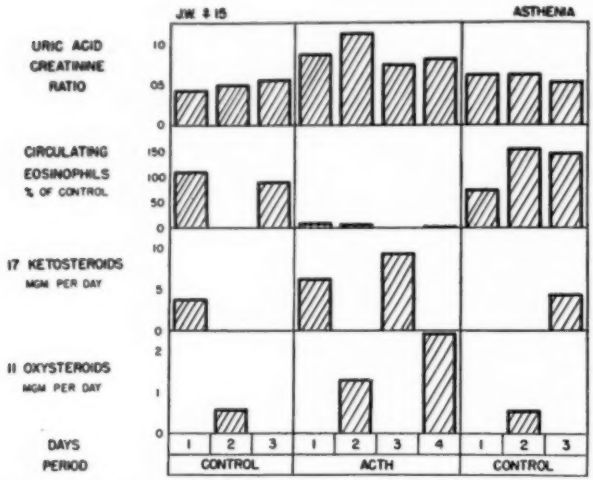


FIG. 5.—Typical responses obtained on prolonged stimulation of the adrenal cortex with 40 mg. daily ACTH. (Reprinted from Forsham *et al.*, 1948.)

Increased Nitrogen Excretion.—Such a change is known to be a constant effect of the administration of 11-oxygenated compounds to animals (Long, Katzin, and Fry, 1940) although variable in amount, being due to the catabolic effect of these hormones (Albright, 1943). The response in nitrogen excretion to ACTH is, however, less constant than might be expected from the results of animal experiments and is more in accord with results observed with administration of "Compound E" in man (Perera, Pines, Hamilton, and Vislocky, 1949).

Uric Acid Metabolism.—After the administration of ACTH there is a consistent increase in the amount of uric acid excreted, though it may decline after several days' treatment (Fig. 5). This phenomenon is also seen as a result of adrenal stimulation due to stress (Pincus *et al.*, 1949) and after adrenal cortical extracts (Babad, 1939). It is thought that uric acid excretion is dependent upon increased production from broken down lymphoid tissue (q.v.) and increased uric acid clearance (Forsham *et al.*, 1948; Hellman, Weston, Escher, and Leiter, 1948), and it has also been suggested by Pincus *et al.* (1949) that under certain conditions brain cell

nucleoprotein may be an additional source. In gouty subjects ACTH is found greatly to increase the clearance of uric acid (Thorn, Bayles, Mansell, Forsham, Hill, Smith, and Warren, 1949). Metabolic data of Forsham *et al.* (1948) have shown that creatinine excretion is not appreciably altered by ACTH, and it has proved convenient to express changes in uric acid excretion as changes in the ratio of uric acid to creatinine (see below).

Changes in Blood Cell Counts.—Following on the extensive work of Dougherty and White (1947) on the lysing action on lymphoid tissue of 11-oxygenated steroids and ACTH in animals, observations in man confirmed these findings (Forsham *et al.*, 1948 ; Hills *et al.*, 1948). With prolonged administration of ACTH there is a large and sustained fall in eosinophil counts and an initial decrease in lymphocyte counts, although the latter is not always maintained. The magnitude of these effects is dependent upon the dosage of ACTH used, but with 40 mg. per day, near zero levels of eosinophils may be attained (Fig. 5). Simultaneously with these changes there is an elevation in the numbers of polymorphonuclears, which may be doubled. This last change was considered to be partly a non-specific one, for some elevation in this count with ACTH was also observed in patients with Addison's disease.

Increased Production of Sodium-retaining Steroids.—Prolonged treatment with ACTH frequently produces extracellular water retention, and sodium and chloride retention (Prunty, Forsham, and Thorn, 1948 ; Forsham *et al.*, 1948). The retention of sodium may be profound ; for instance, on an intake of 74 milliequivalents per day, 64 milliequivalents a day were retained during six days' treatment with 40 mg. ACTH daily. Increased excretion of potassium may occur, due to intracellular loss of this mineral, especially in the early phase after the injection of ACTH. It is considered that these effects on electrolyte balance are indicative of the stimulation of salt-retaining steroids of the adrenal cortex. There are differences observed in the behaviour of different patients in these respects, and it has already been indicated that the response of individuals to desoxycorticosterone may be dependent upon the existing electrolyte and water balance of the body. Potassium excretion may be particularly variable (Elkinton *et al.*, 1949), and ACTH may even produce signs of potassium deficiency.

Increased 17-ketosteroid Excretion.—Large increases in adrenal 17-ketosteroid excretion are observed on prolonged treatment with ACTH (Fig. 5). In a case of mild hypopituitarism the increase in four days on 40 mg. ACTH daily amounted to 500%. This patient showed an increase in the rate of growth of his beard, and had nocturnal erections which had not previously occurred. The nature of the 17-ketosteroids excreted is not likely to lead to increases in the 3 β-hydroxy fraction (Mason *et al.*, 1948).

Failure of Typical Response to ACTH in Adrenal Cortical Insufficiency

In severe adrenal cortical insufficiency none of the changes enumerated above can be expected to occur. That this is the case can be seen from the data in Fig. 6 which is typical of patients with Addison's disease.

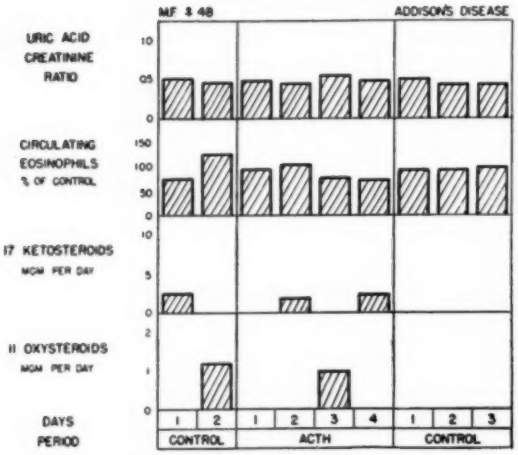


FIG. 6.—Absence of typical responses on prolonged stimulation of the adrenal cortex with ACTH in a patient with Addison's disease. (Reprinted from Forsham *et al.*, 1948.)

Effects of Single Doses of ACTH

On account of the briefer period of stimulation of the adrenal cortex with a single dose of ACTH the response obtained differs in some respects from that with more prolonged administration. Unless large doses are used (Venning *et al.*, 1948) changes in steroid excretion are not measurable. The following paragraphs summarize the effects discernible with a single dose of 25 mg. ACTH in subjects with normally responding adrenal cortices.

Changes in Blood Count.—The characteristic changes enumerated above are also to be seen under these conditions. It has been found that the most uniform results are obtained when the observed changes in blood count are expressed as a percentage variation from the initial count. In patients with intact adrenal cortices the average variation from the resting level four hours after the injection of 25 mg. ACTH were found to be an increase of 100% in polymorphonuclears, a decrease of 40% in lymphocytes (Fig. 7), and a decrease of 75% in eosinophils, with a range from 52 to 98% in 50 individuals, including normal persons and hospital patients. The mechanism of these changes in cell count is not understood. In the case of lympholysis *in vitro* it seems that some component of lymphoid tissue itself is required for the effect to be observed (Hechter and Johnson, 1949). *In vivo* experiments with adrenal steroids have shown that the effect on eosinophils is due to secretion of 11-oxygenated steroid by the adrenal cortex in response to ACTH. The administration of approximately 20 mg. of "Compound F" to seven patients with Addison's disease resulted in an average fall of eosinophils of 61%, whereas neither desoxycorticosterone nor testosterone in the form of easily absorbable water soluble compounds had any appreciable effect in doses of approximately 30 mg. The time relationship of the response in blood count to the administration of ACTH is shown in Fig. 7. The maximum fall in lymphocytes is seen to occur four hours after the dose of ACTH has been given, and this too is the time of maximum response to eosinophils. The same finding was observed after the administration of "Compound F." This suggests that the time interval involved in the response of the adrenal cortex to ACTH is small and that most of the intervening period is concerned with the action of the steroid on the cells. It is difficult to press this argument too far, however, for ACTH is known to be very rapidly absorbed, whereas the absorption of "Compound F" is more prolonged.

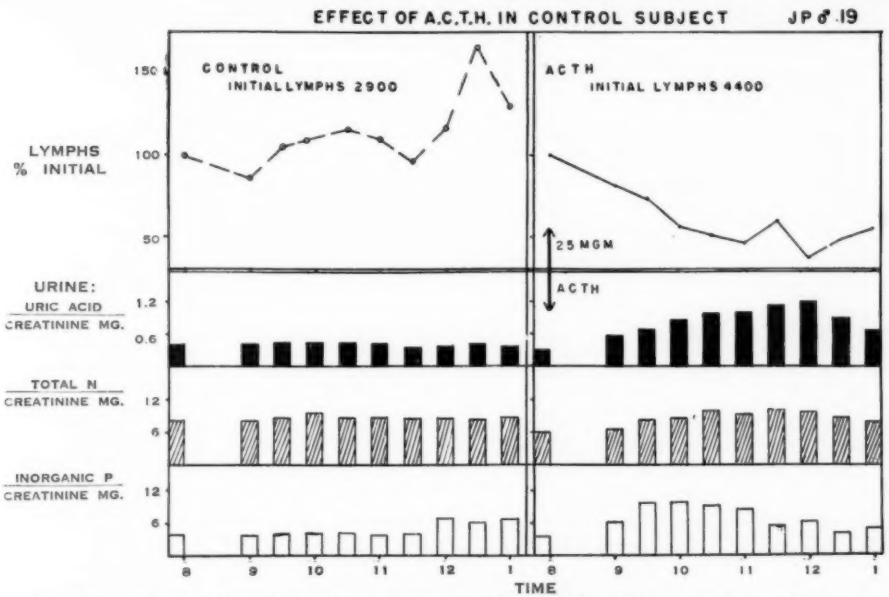


FIG. 7.—The time relations of lymphocyte response and uric acid excretion to injection of 25 mg. ACTH I.M. (Reprinted from Thorn and Forsham, 1949.)

Effect on Uric Acid Excretion.—In Fig. 7 the results of the administration of 25 mg. of ACTH to a normal individual are shown. Uric acid and creatinine excretion were measured in urine specimens obtained at half-hourly intervals. It will be seen that there is relatively small change in the creatinine, but a sharp increase in the uric acid excretion, which was maximal at four hours, coincident with the greatest change in blood count. It takes at least one hour before any measurable change occurs. In the same way that blood eosinophils are affected by "Compound F" so is the increase in uric acid excretion. Twenty mg. of "Compound F" given to two patients with Addison's disease caused an increase over the pre-injection level of 48% and 45% in the ratio uric acid: creatinine (uric acid and creatinine in the urine being expressed in mg. %). In a third patient the ratio increased 180% during seven hours following injection of 17 mg. of "Compound F." Desoxycorticosterone in a dose of 30 mg. produced a small but definite increase in the ratio in two patients (25%).

Alterations in Electrolyte Excretion.—Increases in the amounts of sodium, chloride, and potassium excreted during the four hours following injection of 25 mg. of ACTH have consistently been observed and have been related to the similar effect produced by 11-oxygenated steroids (Forsham *et al.*, 1948). These observations form a contrast to those with the more prolonged administration of ACTH considered above. Apparently in short term observations the effect of 11-oxygenated steroids is predominant.

The Use of ACTH in Testing Adrenal Cortical Function

The use of a single test dose of ACTH provides information about the capability of the adrenal to give an immediate response, while the more prolonged 48-hour test to be described allows the adrenal a greater opportunity of giving a measurable response under certain conditions. From the data reviewed above it will be seen that the two most useful and convenient measurements that can be made in a short test are the change in eosinophil count and increase in uric acid excretion. The former has the advantage, unlike so many other indices of adrenal cortical function, that it is independent of the kidney as a mediator of the response. It suffers from the disadvantage that certain individuals with allergic eosinophilia may fail to respond (Thorn and Forsham, 1949) and occasionally patients are encountered with eosinopenia due to bone marrow depression. The measurement of uric acid excretion has been found to be less reliable than changes in eosinophil count. If the uric acid excretion is approaching a maximum due to the presence of gout, leukaemia, or decrease of renal function, or in some instances to an increase of adrenal cortical secretion, it may be difficult further to increase the excretion of uric acid. Secondly, it seems that other factors which are not understood may at times interfere with the uric acid responses. Increased uric acid excretion may, therefore, be regarded as valuable corroborative evidence of adequate adrenal cortical activity, if it is present. The response in uric acid excretion is also probably a less sensitive index of adrenal stimulation than changes in the eosinophil count.

The Four-hour ACTH Test.—The technique adopted for the four-hour ACTH test by Forsham *et al.* (1948) may be restated as follows:—

The patient is allowed no breakfast (glucose ingestion is known to increase uric acid excretion) but water is freely permitted, and 200 ml. are given at the start of the test. At this time urine is voided and two hours later voided again and this control specimen kept. Then 25 mg. ACTH (equivalent to the Armour Laboratory standard) are injected intramuscularly, 200 ml. water given again, and the latter is repeated two hours later. One hour after the ACTH injection the patient voids and the urine is rejected. The urine passed during the subsequent three hours is collected. Eosinophil counts are carried out immediately before the injection of

TABLE IV

DEGREES OF RESPONSE TO ACTH

Patient			Diagnosis	Chemical Degree of Pituitary Insufficiency	Eosinophil Change (%)	Uric Acid Creatinine Change (%)
No.	Sex	Age				
*1	F	42	Simmonds's disease	Severe	+24	+1
2	F	33	,, ,,	,,	−4	+18
3	F	61	,, ,,	Moderate	−17	+22
4	M	58	Chromaphobe adenoma	Severe	−15	+32
5	M	60	,, ,,	Mild	−27	+49
6	M	46	,, ,,	,,	−45	+90
7	M	40	,, ,,	,,	−32	+39

* The data on Cases 1 to 6 are taken from the article by Forsham *et al.* (1948); Case 7 is unpublished.

ACTH and at the end of four hours after this time. Uric acid and creatinine are determined in the two urine specimens and expressed as mg.%.*

In patients with normal adrenal cortical function a fall of 50% or more in the eosinophil count is observed, and an increase of 50% or more in the uric acid: creatinine ratio indicates a good response (Fig. 8). In patients with Addison's disease these minimum changes are not attained. Patients having adrenal cortical failure secondary to anterior pituitary deficiency may show varying degrees of response (Table IV) depending upon the severity of their condition and the degree of secondary adrenal cortical atrophy.

The 48-hour ACTH Test.—This test, described by Thorn and Forsham (1949), has the advantage that it allows a longer time for stimulation of the adrenal cortex. It is also favoured by the fact that measurable changes in 17-ketosteroid excretion can be observed and the specificity of the test thereby improved. The patient receives a total dose of 40 mg. of ACTH per day in doses of 10 mg. at six-hourly intervals, day and night for two days. The eosinophil count is measured before the first dose of ACTH and four hours after the last dose. A 24-hour specimen of urine is collected the day preceding the first dose of ACTH and a second one during the last 24 hours of ACTH injection. The authors state that a low initial 17-ketosteroid excretion which fails to rise after ACTH accompanied by a poor fall in eosinophils (less than 50%) indicates absence of adrenal cortical reserve as is seen in Addison's disease and hypopituitarism. A low initial 17-ketosteroid excretion which rises after ACTH with a progressive fall in eosinophils is suggestive of moderate adrenal cortical deficiency due to pituitary failure, for example, while a normal 17-ketosteroid excretion followed by a rise to the upper limit of normal indicates a normal reserve function of the cortex. A fall of 50% or more in the eosinophils is corroborative evidence for this. They cite examples of cases illustrating these points, and it is interesting to note that a patient with acromegaly and two patients with Cushing's syndrome gave supernormal responses, the 17-ketosteroid levels rising to 31, 53, and 119 mg. per day respectively. This is good evidence for the claim that the rise in 17-ketosteroids can be regarded as an index of cortical capacity.

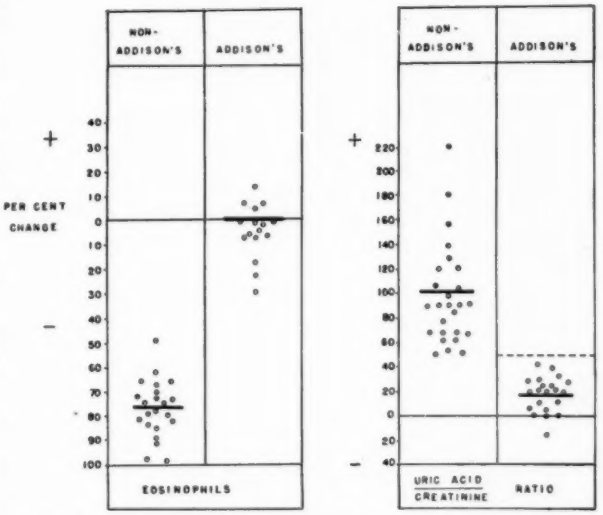


FIG. 8.—Scatter diagram of responses of patients with normal adrenal cortical function and patients with Addison's disease to 25 mg. ACTH. (Reprinted from Prunty, 1949b.)

* The method used for uric acid determination is given in the appendix.

Responses of the Adrenal Cortex to Adrenaline and to Stress Stimuli

The eosinopenic action of adrenaline was first noted by Bertelli, Falta, and Schweeger (1910) in dogs. In the light of the experiments reviewed earlier in this paper it can be seen how this effect is likely to be mediated through the secretion of adrenal cortical 11-oxygenated steroids. For this reason a test for the integrity of the adrenal cortex, combined with that of the anterior pituitary, based on the action of adrenaline has been proposed (Recant, Hume, Forsham, and Thorn, 1950 ; Thorn and Forsham, 1949 ; Thorn, Bayles, *et al.*, 1949). A subcutaneous dose of 0.3 mg. of adrenaline is injected and the eosinophils counted immediately before the dose and four hours later. Normal individuals respond with a fall of 50% or more in the count. Neither patients with hypopituitarism nor Addison's disease respond to this test in the normal way. The data given by the authors show good correlation with the 4-hour ACTH test, but the latter is preferred. Clearly with the scarcity of ACTH in England this test is worth further trial.

The characteristic response of the adrenal to stress is also accompanied by eosinopenia. This may prove of value in assessing the response of patients to trauma and operations. Poor responses may be expected with anterior pituitary or adrenal cortical insufficiency, and in poor states of nutrition (Venning and Browne, 1949). Thorn and Forsham (1949) have found that after severe pain, injury, infection, and operation there is normally a large depression in eosinophil counts. It would be expected that patients with Addison's disease and hypopituitarism would fail to show this change. In patients with mild degrees of the latter post-operative reactions may be poor, but administration of ACTH for 48 hours should bring about a normal response. Such tests may prove of considerable value in assessing the condition of these patients and in determining in which instances the adrenal cortex has become exhausted and fails to respond.

Summary

Abnormalities of adrenal cortical function may be found in a wide range of conditions. These abnormalities may be detected by tests designed to assess the resting function of the gland, or tests based upon the capacity of the gland to react to various stimuli, which give an indication of the functional reserve. These latter tests depend upon the stimulation of the cortex either by exogenous or endogenous ACTH. The physiological properties of ACTH have been reviewed and it has been shown how these may be applied in the use of ACTH as a test for adrenal cortical functional reserve. Brief consideration has also been given to the behaviour of the adrenal cortex in conditions of stress, such as the post-operative state.

I am indebted to Charles C. Thomas, publisher, U.S.A., for permission to reproduce Figs. 5 and 6 ; to the Academic Press Inc., New York, for permission to reproduce Fig. 7 ; and to the Editor of the *Proceedings of the Royal Society of Medicine* for permission to reproduce Fig. 8.

References

Albright, F. (1943). *Harvey Lect.*, **38**, 123.
Babad, P. (1939). *Arch. int. Physiol.*, **49**, 327.
Bertelli, G., Falta, W., and Schweeger, O. (1910). *Z. klin. Med.*, **71**, 23.
Callow, R. K. (1938). *Proc. R. Soc. Med.*, **31**, 841.

Callow, N. H., Callow, R. K., and Emmens, C. W. (1938). *Biochem. J.*, **32**, 1312.
—— and Crooke, A. C. (1944). *Lancet*, **1**, 464.
Collip, J. B., Anderson, E. M., and Thomson, D. L. (1933). Ibid., **2**, 347.
Conn, J. W., Louis, L. H., and Johnston, M. W. (1949). *J. Lab. clin. Med.*, **34**, 255.
Crooke, A. C., and Callow, R. K. (1939). *Quart. J. Med.*, **8**, 233.
Cutler, H. H., Power, M. H., and Wilder, R. M. (1938). *J. Amer. med. Ass.*, **111**, 117.
Daughaday, W. H., Jaffe, H., and Williams, R. H. (1948a). *J. clin. Endocrinol.*, **8**, 166.
—— —— —— (1948b). Ibid., **8**, 244.
de la Balze, F. A., Reifenstein, E. C., and Albright, F. (1946). *J. clin. Endocrinol.*, **6**, 312.
Dorfman, R. I. (1948). *Recent Progress in Hormone Research*, **2**, 179.
Dougherty, T. F., and White, A. (1947). *J. Lab. clin. Med.*, **32**, 584.
Dunger, R. (1910). *Münch. med. Wschr.*, **57**, 1942.
Elkinton, J. R., Hunt, A. D., Godfrey, L., McCrory, W. W., Rogerson, A. G., and Stokes, J. (1949). *J. Amer. med. Ass.*, **141**, 1273.
Evans, H. M. (1923–4). *Harvey Lect.*, p. 212.
Folin, O. (1914). *J. biol. Chem.*, **17**, 469.
Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G. (1948). *J. clin. Endocrinol.*, **8**, 15.
Fraser, R. W., Forbes, A. P., Albright, F., Sulkowitch, H., and Reifenstein, E. C. (1941). Ibid., **1**, 234.
Gaunt, R. (1946). Ibid., **6**, 595.
Haines, W. J., Johnson, R. H., Brunner, M. P., Pabst, M. L., and Kuizenga, M. H. (1949). *Fed. Proc.*, **8**, 203.
Heard, R. D. H., and Sobel, H. (1946). *J. biol. Chem.*, **165**, 687.
—— —— and Venning, E. H. (1946). Ibid., **165**, 699.
Hellman, L., Weston, R. E., Escher, D. J. W., and Leiter, L. (1948). *Fed. Proc.*, **7**, 52.
Hechter, O., and Johnson, S. (1949). *Endocrinology*, **45**, 351.
Hills, A. G., Forsham, P. H., and Finch, C. A. (1948). *Blood*, **3**, 755.
Hoffman, L. M. (1944). *J. clin. Endocrinol.*, **4**, Suppt., 215.
Hume, D. M. (1949). *J. clin. Invest.*, **28**, 790.
Kepler, E. J., and Mason, H. L. (1947). *J. clin. Endocrinol.*, **7**, 543.
—— Sprague, R. G., Mason, H. L., and Power, M. H. (1948). *Recent Progress in Hormone Research*, **2**, 345.
Kern, A., and Stransky, E. (1937). *Biochem. Z.*, **290**, 419.
Krebs, H. A., and Örström, A. (1939). *Biochem. J.*, **33**, 984.
Levy, M. S., Power, M. H., and Kepler, E. J. (1946). *J. clin. Endocrinol.*, **6**, 607.
Li, C. H. (1950). *Brit. med. J.*, **1**, 299.
—— Evans, H. M., and Simpson, M. E. (1943). *J. biol. Chem.*, **149**, 413.
Lieberman, S., Dobriner, K., Hill, B. R., Fieser, L. F., and Rhoads, C. P. (1948). Ibid., **172**, 263.
Long, C. N. H. (1947). *Recent Progress in Hormone Research*, **1**, 99.
—— Katzin, B., and Fry, E. G. (1940). *Endocrinology*, **26**, 309.
Mason, H. L. (1948). *Recent Progress in Hormone Research*, **3**, 103.
—— Power, M. H., Rynearson, E. H., Ciaramelli, L. C., Li, C. H., and Evans, H. M. (1948). *J. clin. Endocrinol.*, **8**, 1.
—— and Sprague, R. G. (1948). *J. biol. Chem.*, **175**, 451.
Morris, P., and Morris, C. J. O. R. (1950). *Lancet*, **1**, 117.
Munson, P. L., Jones, M. E., McCall, P. J., and Gallagher, T. F. (1948). *J. biol. Chem.*, **176**, 73.
Perera, G. A., Pines, K. L., Hamilton, H. B., and Vislocky, K. (1949). *Amer. J. Med.*, **7**, 56.
Pincus, G., Hoagland, H., Freeman, H., and Elmadjian, F. (1949). *Recent Progress in Hormone Research*, **4**, 291.
Prunty, F. T. G. (1949a). *J. clin. Invest.*, **28**, 690.
—— (1949b). *Proc. R. Soc. Med.*, **42**, 267.
—— Forsham, P. H., and Thorn, G. W. (1948). *Clin. Sci.*, **7**, 109.
Recant, L., Hume, D. M., Forsham, P. H., and Thorn, G. W. (1950). *J. clin. Endocrinol.*, **10**, in press.
Reinecke, R. M., and Kendall, E. C. (1943). *Endocrinology*, **32**, 505.
Reichstein, T., and Shoppee, C. W. (1945). *Vitamins and Hormones*, **1**, 346.
Robinson, F. J., Power, M. H., and Kepler, E. J. (1941). *Proc. Mayo Clin.*, **16**, 577.
Sayers, G., Burns, T. W., Tyler, F. H., Jager, B. V., Schwartz, T. B., Smith, E. L., Samuels, L. T., and Davenport, H. W. (1949). *J. clin. Endocrinol.*, **9**, 593.
—— and Sayers, M. A. (1949). *Ann. N.Y. Acad. Sci.*, **50**, 522.
—— White, A., and Long, C. N. H. (1943). *J. biol. Chem.*, **149**, 425.
Selye, H. (1946). *J. clin. Endocrinol.*, **6**, 117.
Smith, P. E. (1930). *Amer. J. Anat.*, **45**, 205.
Soffer, L. J., Gabrilove, J. L., and Jacobs, M. D. (1949). *J. clin. Invest.*, **28**, 1091.
Speirs, R. S., and Meyer, R. K. (1949). *Endocrinology*, **45**, 403.

Talbot, N. B., Albright, F., Saltzman, A. H., Zygmuntowicz, A., and Wixom, R. (1947). *J. clin. Endocrinol.*, **7**, 331.
—— Butler, A. M., and Maclachlan, E. A. (1940a). *New Engl. J. Med.*, **223**, 369.
—— —— —— (1940b). *J. biol. Chem.*, **132**, 595.
—— Saltzman, A. H., Wixom, R. L., and Wolfe, J. K. (1945). Ibid., **160**, 535.
Thorn, G. W., Bayles, T. B., Massell, B. F., Forsham, P. H., Hill, S. R., Smith, S., and Warren, J. E. (1949). *New Engl. J. Med.*, **241**, 529.
—— and Forsham, P. H. (1949). *Recent Progress in Hormone Research*, **4**, 229.
—— —— Prunty, F. T. G., Bergner, G. E., and Hills, A. G. (1949). *Ann. N.Y. Acad. Sci.*, **50**, 646.
Venning, E. H., and Browne, J. S. L. (1949). Ibid., **50**, 627.
—— Kazmin, V. E., and Bell, J. C. (1946). *Endocrinology*, **38**, 79.
—— —— Ripstein, M., McAlpine, H. T., and Hoffman, M. M. (1948). *J. clin. Endocrinol.*, **8**, 605.
Vogt, M. (1944). *J. Physiol.*, **103**, 317.
—— (1945). Ibid., **104**, 60.
Warren, F. L. (1945). *Cancer Res.*, **5**, 49.
Willson, D. M., Robinson, F. J., Power, M. H., and Wilder, R. M. (1942). *Arch. intern. Med.*, **69**, 460.

APPENDIX

Eosinophil Counts

Venous blood, 5 ml., is drawn with a syringe and placed in an oxalate bottle and gently mixed. The bottle is prepared by drying off in it 0.5 ml. solution containing 0.8 g. potassium oxalate and 1.2 g. ammonium oxalate per 100 ml. The blood is drawn up to the 0.5 mark in a white cell pipette and diluted with fluid to the 11 mark. The diluting fluid (Dunger, 1910) contains 5 ml. 2% aqueous eosin, 5 ml. acetone, and 90 ml. distilled water, and is filtered before use. The pipette is gently agitated for 30 seconds and some of the fluid, discarding the first few drops, placed on a Fuchs-Rosenthal chamber. The cells covering the field are counted after 2 minutes. As many counts as possible should be made, employing freshly diluted blood for each. If four chambers are counted the number of eosinophils per $mm.^3$ is given by the total count multiplied by 0.625.

Uric Acid Estimation in Blood and Urine

This has been modified from the method described by Kern and Stransky (1937) (see also Krebs and Örström, 1949).

Reagents

The reagents present are listed below, and it should be specially noted that the solution of sodium silicate and glycerol is critical since some brands lead to the formation of opalescence during colour development.

1. 0.25 N Sodium Hydroxide.

2. Uric Acid Reagent.—Sodium tungstate, 50 g., which must be molybdenum-free,* is dissolved in 400 ml. water; 40 ml. phosphoric acid (gr. 1.75) are added from a cylinder and the cylinder washed out. The mixture is boiled under reflux for two hours, cooled, and made up to 500 ml. It should be stored in a brown bottle.

3. Silicate-glycerol Reagent.—Sodium silicate.† 230 g., Sp.g.1.7, is dissolved in 300 ml. hot water and filtered. Glycerol, 85 ml., is added (B.D.H.—"reagent grade," not AR) and made up to 500 ml.

4. Liquoid-uric Acid Reagent.—Liquoid, 2.78 g., is dissolved‡ in 139 ml. water and 111 ml. uric acid reagent added. Solution is made up to 500 ml. with water.

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* Eimer and Armand, N.Y. † General Chemical and Pharmaceutical Co., Ltd. ‡ Roche.

5. Uric Acid Standard Solution (1 mg./1 ml.).—Dry, pure uric acid, 1 g., is put into a 1,000 ml. flask through a dry funnel, and 0.6 g. lithium carbonate dissolved in 150 ml. water by warming to 40° C. and filtered. The 1,000 ml. flask is warmed to 50°–60° C. and the lithium carbonate poured in and washed in. It is kept warm for 5 minutes until dissolved. It is cooled at once and 20 ml. 40% formalin added. The flask is half filled with water and a few drops methyl orange added. *N* sulphuric acid, 25 ml., are run in until methyl orange goes pink. (There should be 2–3 ml. of acid left in the pipette.) The remainder of the 25 ml. of acid is then run in, mixed, and made up to 1,000 ml. For a working standard 1 ml. is diluted to 200 ml.

Technique

Plasma.—To 1 ml. of plasma is added 8 ml. water and 0.4 ml. NaOH; 0.6 ml. uric acid reagent is added slowly, the solution allowed to stand 10 minutes and spun; 5 ml. supernatant is taken and treated as for urine.

Urine.—Dilute 1 ml. to 100 ml. (or 1/200 or 1/50 as necessary). Uric acid solution 5 ml. (standard, plasma filtrate, or diluted urine) are treated with 2.5 ml. silicate-glycerine solution and 2 ml. liquoid-uric-acid-reagent, the mixture allowed to stand for 6 minutes, and read in the photoelectric-colorimeter. Optimum absorption is at 700 mμ.

Calculation

$$\textit{Plasma} \quad 5 \times \frac{U}{S} = \text{mg.\% uric acid}$$

$$\textit{Urine} \quad 50 \times \frac{U}{S} = \text{mg.\% for 1/100 dilution.}$$

With this technique normal plasma values vary between 2.5 and 6 mg. %, and fasting levels of uric acid: creatinine ratio average 0.46, S.D. ± 0.092. The specificity for this reaction in urine has been shown to be over 90% when uricase is employed to destroy the uric acid in urine specimens (Forsham *et al.*, 1948).

For methods of creatinine estimation in urine see Folin (1914).

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THE SERUM TRYPTOPHANE-PERCHLORIC ACID REACTION AS A MEASURE OF TISSUE DESTRUCTION

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Increases in the level of serum polysaccharide have been found in a number of diseases (Lustig and Langer, 1931 ; Nilsson, 1937 ; Blix, Tiselius, and Svensson, 1941 ; Seibert, Seibert, Atno, and Campbell, 1947), in particular in tuberculosis and carcinoma, and it has been suggested (Seibert *et al.*, 1947) that this increase is associated with tissue destruction—a conclusion in keeping with the finding of Lustig, Leiner, and Ernst (1937) that the carbohydrate content of the serum proteins in venous blood was generally higher than in arterial. In view of the possibility that this serum carbohydrate is derived from nucleic acid, Seibert, Pfaff, and Seibert (1948) applied to serum the tryptophane-perchloric acid (TA) reaction of Cohen (1944), when they found a significant increase in the reaction of cases of tuberculosis and several types of carcinoma. The nature of the reacting substance is unknown, but Seibert *et al.* have produced evidence suggesting that it is derived from proteins. This increased serum TA reaction might also be associated with tissue destruction.

It seemed of interest to test this hypothesis by applying the TA reaction to serum obtained from burned patients, in whom tissue destruction is known to occur, and where possible correlating the result with the excretion of nitrogenous substances in the urine. A preliminary report of the main findings has been published (Keyser, 1949).

Methods

Blood was obtained from the burned patients by venepuncture as soon as possible after admission to hospital, and thereafter at intervals. After clotting had occurred the serum was separated and kept frozen until the TA test could be performed. Under these conditions sera did not deteriorate for several weeks at least. Haemolysed specimens were not used for this test. The TA reaction was carried out as described by Seibert *et al.* (1948), except that the quantities of reagents (but not the proportions) were altered slightly for convenience.

Serum, 0.5 ml., was heated with 3.5 ml. 0.4% saline, 2.0 ml. 0.5% tryptophane, and 6 ml. 60% perchloric acid in a stoppered glass tube for exactly 10 minutes in a vigorously boiling water bath. After standing in ice-water for 40 minutes with occasional shaking the solution was filtered through a No. 5 Whatman filter paper and the extinction coefficient measured with the Spekker absorptiometer, using filter

No. 6* and a 1 cm. cell. A reagent blank was run with each series of determinations. Where possible a serum blank was allowed for by diluting 0.5 ml. serum with 11.0 ml. saline and 0.5 ml. 2.5*N*.NaOH (Seibert, private communication). Results were recorded as (Spekker reading – blanks) × 100, i.e.

$$E_{1\text{ cm.}}\ (\text{corr.}) \times 100$$

Readings were almost exactly proportional to the amount of serum taken for the test. Where transfusions of plasma, glucose, or saline were given, specimens of the transfusion fluids were subjected to the TA test: they were found not to give the reaction. Serum proteins were determined by the micro-Kjeldahl method. Non-protein nitrogen was determined in two cases by the method of King, Haslewood, and Delory (1937) or by Kjeldahl analysis of the Folin-Wu filtrate.

The TA test controls for Group 1 cases were eight children of about the same age who had been admitted for the investigation of minor complaints or the repair of defects such as cleft palate, but who were apparently normal in other respects. The controls for Group 2 were 10 normal adults.

In the case of the adult patients, 24-hour collections of urine were made, with chloroform as preservative, but owing to the absence of facilities for accurate metabolic work it is possible that some of these urine collections were in fact incomplete. Urine was tested for protein, sugar, and acetone bodies, and the nitrogenous constituents were determined as follows: total nitrogen by the Kjeldahl method, creatine and creatinine by Folin's (1914) method, and uric acid by the modification of the method of Benedict and Franke (1922), described by King (1946).

It was not always possible to follow up the cases for more than a few days owing to the patients' discharge from hospital.

Technical Study of the TA Test

Three main difficulties were encountered in applying the TA reaction. In the first place, since the identity of the reacting substance is unknown it is difficult to control the reaction except by reference to some arbitrary standard such as fructose, or by using specimens of serum as reference standards from time to time and also when changing from one batch of reagents to another. Secondly, it is impossible to carry out true serum blank determinations on account of the presence of tryptophane in the serum proteins. According to Seibert *et al.*, variations in the natural colour of sera are allowed for by making control solutions of the sera in saline (Seibert *et al.*, 1948) or in saline and NaOH (Seibert, private communication). Whether or not such a procedure gives a true serum blank, it was found in practice that many sera gave slightly opalescent solutions, even in the presence of NaOH, and in such cases an arbitrary blank of $E_{1\text{ cm.}} \times 100 = 1.5$ was subtracted from the test reading. (This was an average blank obtained with non-lipaemic sera: very little difference was found even with fairly deeply pigmented, but non-haemolysed, sera, provided that their solutions in saline and NaOH were clear.)

The third, and most serious, difficulty encountered was that the amounts of colour developed in the test varied from one batch of perchloric acid to another; furthermore some batches gave high reagent blanks and poor colour development and were

*Subsequent work showed that somewhat higher readings are obtained with the No. 7 filter.

therefore rejected. (Only AnalaR* perchloric acid was used: it conformed to the AnalaR specifications for content of $HClO_4$ and absence of chloride and chlorate.) These blank values cannot be allowed for simply by subtraction from the test reading, as the substance responsible for the blank value seems actually to inhibit colour development in the test, as is shown by some typical results given in Table I.

TABLE I

INHIBITION OF COLOUR DEVELOPMENT BY REAGENT BLANK IN THE TA TEST

Perchloric Acid	Serum JWK			Serum TVB		
	Test*	Blank*	Difference*	Test*	Blank*	Difference *
1	14.2	2.1	12.1	14.2	2.1	12.1
2	16.8	8.2	8.6	17.5	8.2	9.3

* $E_{1\,cm.} \times 100$

In changing from one batch of reagents to another it is therefore necessary to check the amount of colour developed with each lot, using the same sera. In this way a correction may be applied, enabling results obtained with one lot of reagents to be related to those obtained with another.

It was confirmed that glucose gives a negligible amount of colour in the test. Urea, uric acid, and creatinine give no colour.

Results in Burn Cases

In this investigation the cases are divided into two groups (Cases 1–4 and 5–7) in which two different batches of perchloric acid were used. On account of a long delay, during which it was not possible to obtain satisfactory perchloric acid, the two groups are unrelated, but each group has its own series of appropriate normal values. The same batch of tryptophane was used throughout.

In spite of our criticisms of the TA test it is clear that after burning there is generally a more or less pronounced rise in the extinction coefficient in the TA test (Figs. 1 and 2), often up to values of the order met with in cancer. With the exception of Case 4, the TA extinction coefficient, at the peak of the rise, was 32% to 156% above the initial value, which may probably be taken as approximating to the normal, pre-burn level. (It is assumed that in Case 3, where it was not possible to obtain a blood specimen before the third day, the initial value would have been 7.4—see Fig. 1.) In one case, not shown in the figures, a single specimen of serum obtained from a child two days after burning gave a normal result (Case 4). Here it is possible that an initial slight rise was missed.

Spectrophotometric measurement of the absorption spectra of the TA reaction mixtures obtained with sera from two normal people, two burn cases, and two carcinoma cases showed the same absorption maxima (Fig. 3). For clarity, only one curve from each pair is shown in the figure.

* By the co-operation of Messrs. The British Drug Houses, Ltd., and Messrs. Hopkin and Williams, Ltd., we were able to obtain special batches of AnalaR perchloric acid which had been specially tested and shown to give satisfactory colour development.

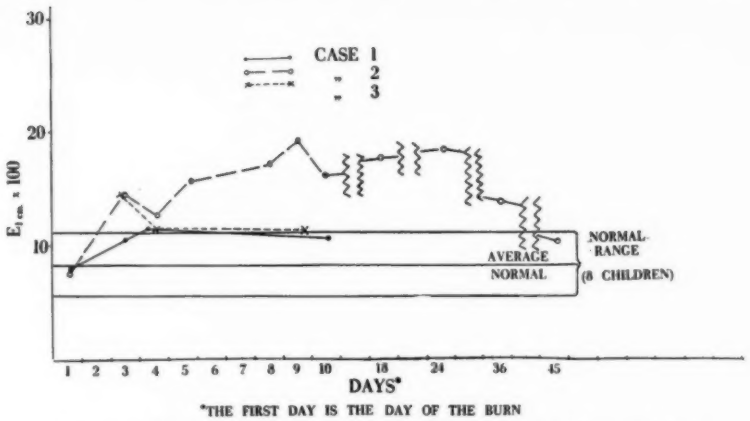

FIG. 1.—Rise in extinction coefficient in TA test after burning (Group 1 cases).

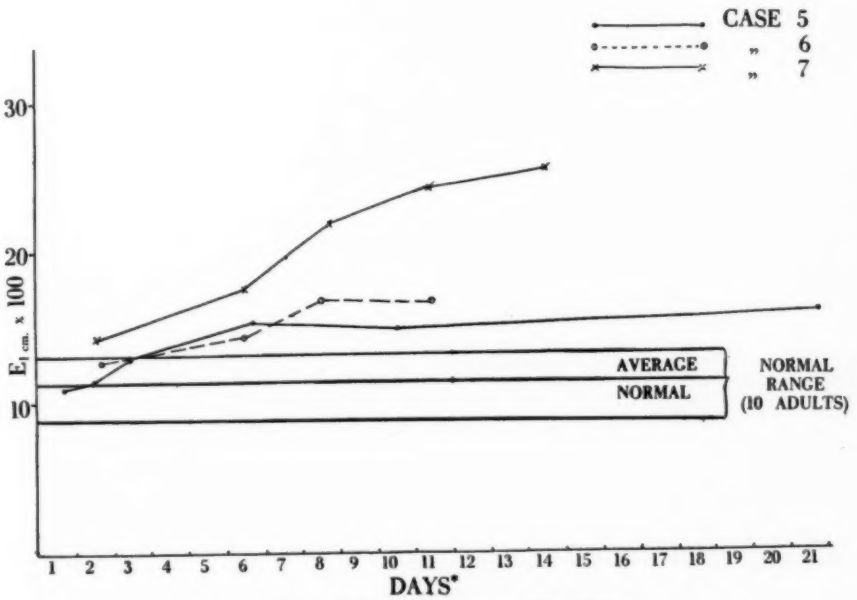
CASE 5
" 6
" 7
30
20
10
$E_{1\,cm.} \times 100$
AVERAGE NORMAL
NORMAL RANGE (10 ADULTS)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
DAYS*
*THE FIRST DAY IS THE DAY OF THE BURN

FIG. 2.—Rise in extinction coefficient in TA test after burning (Group 2 cases).

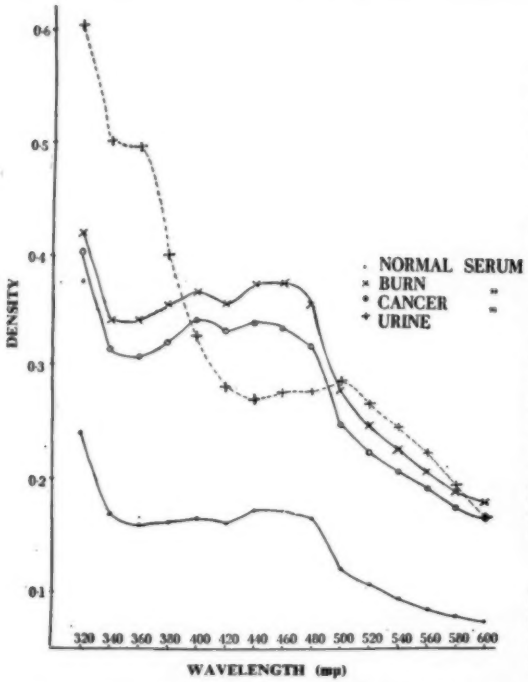

FIG. 3.—Absorption curves of reaction mixtures in TA test.

Case Notes

The following notes give necessary clinical details. Serum protein and non-protein nitrogen values are given in Table II.

Case 1.—V. B., a girl of 2½ years, was admitted with scalds (second degree) extending from the interscapular region to the buttocks and on a small area of the left heel. The burned area was cleaned with "cetavlon" and treated with penicillin and sulphonamide powder. Transfusions included 400 ml. plasma, 500 ml. plasma-saline, and 1,000 ml. plasma-glucose. Penicillin was given (50,000 units three-hourly) until the sixth day. Other drugs given were "nembutal," nepenthe, and chloral.

Case 2.—K. M., a girl aged 2 years, was admitted with first and second degree burns of the face, chest, and arms sustained when she fell on to an open fire and her clothes caught fire. The burns were treated with sulphonamide and penicillin powder. A total of 800 ml. plasma and 200 ml. glucose-saline was transfused. Penicillin was given (25,000 units three-hourly) until about the fortieth day, by which time the TA reaction had decreased to a nearly normal level. The temperature was barely above normal after the second day. Other drugs used were nepenthe, ephedrine, protargol (nasal drops), "nembutal," chloral.

Case 3.—L. P., a girl aged 2½ years, was admitted with first degree scalds of the chest and upper arms, and a few areas of second degree scalding, received two days previously. Penicillin was injected (50,000 units three-hourly) until the seventh day, but no plasma transfusions were given. The temperature was normal after about the second day. Nepenthe was also used in this case.

Case 4.—B. C., a boy of 3½ years, was admitted with burns of the chest (first degree with small areas of second). The TA test, performed two days later, gave a normal result.

Case 5.—G. C., a male adult, was admitted with second degree burns of the face, hands, and left thigh, caused by a petrol fire. The burns were treated with sulphonamide and penicillin powder, and systemic penicillin (100,000 units three-hourly) was given up to the twenty-fifth day. The patient's temperature was normal after the second day. Other drugs used in this case were morphine and atropine.

TABLE II

SERUM PROTEIN VALUES IN CASES 1, 2, 3, 5, 6, AND 7

Case No.	Day*	Serum Protein (g./100 ml.)	Non-protein N (mg./100 ml.)
1	1	6.1	—
	3	7.3	—
	10	6.8	—
2	1	6.5	34
	3	6.7	26
	5	6.2	35
	9	6.5	32
	14	6.8	—
	45	6.9	—
3	1	6.3	—
	6	5.8	—
5	1	6.9	—
	3	6.5	—
	6	5.5	—
6	2	6.8	—
	6	6.2	—
	8	6.9	—
	11	7.1	—
7	2	6.0	42
	6	6.0	25
	8	6.2	32
	14	6.9	29

* The day of the burn is taken as the first day.

Case 6.—D. C., a man aged 30, was admitted with second degree burns of face, ears, and hands, sustained in a gas explosion. "Cetavlon" was used to clean the burns, which were treated with penicillin and sulphonamide. Systemic penicillin (100,000 units three-hourly) was given up to the tenth day. The temperature was normal after the first day. No plasma was transfused. Morphine was also given after admission.

Case 7.—R. H., a man of 59, was admitted with extensive third degree burns of face, ears, and both hands, with second degree burns also. (This patient was injured in the same gas explosion as Case 6.) The usual treatment was given with "cetavlon," penicillin and sulphonamide, and intravenous plasma; penicillin was given systemically up to the eighteenth day (100,000 units three-hourly). The temperature became normal after the twelfth day. The chief complication in this case was sepsis, and the principal accessory chronic disease was stated to be chronic bronchitis and emphysema.

Discussion

The observations reported are consistent with the view that the TA reaction provides a measure of tissue destruction.

With the exception of Case 7, all the patients were free from generalized infection, though even in Case 7 it is clear that there was a pronounced increase in the TA

reaction from the second to the fourteenth day. This rise is unlikely to have been due to the bronchitis and emphysema already present, the first value obtained being only slightly above the upper normal level (see Fig. 2).

It is difficult to draw any conclusions from the urinary analyses, especially in view of the very small number of cases. Too much reliance cannot be placed on the figures for total amounts excreted, and the full results are therefore omitted from this account. It may be significant, however, that of the two cases (6 and 7) studied side by side, the more severely burned patient showed the greater excretion of creatine and total nitrogen as well as a more pronounced rise in TA reacting substance. Thus Case 6 excreted only about 45 mg. creatine from admission up to the eighth day, though there was then a slight increase, 83 mg. being excreted from the eighth to ninth days. Case 7, however, excreted about 900 mg. of creatine in the first five days.

Clark, Peters, and Rossiter (1945) and Croft and Peters (1945) found that after a severe burn rats showed a sharp rise in creatine excretion. Cuthbertson, McGirr, and Robertson (1939) in experiments on rats showed that fracture of the femur caused a definite loss of creatine in the urine that paralleled nitrogen excretion, and Cuthbertson (1936) reported similar findings in a study of human fractures. I (Keyser, 1947, 1948) reported that three adult males with burns of 20, 15, and 7% of the body surface, respectively, excreted creatine in significant amounts, whereas four other adult males with burns of not more than 2½% of the body surface excreted little or none. The excretion of creatine appears to result from bodily injury and consequent tissue destruction. Creatinuria has been reported as occurring in normal healthy males (see, e.g., Hobson, 1939), though other workers, in extended studies, have failed to confirm this (Tierney and Peters, 1943).

Uric acid excretion, where measured, was within normal limits (Cases 5, 6, and 7).

A point of great interest is that Seibert *et al.* (1948) found a correlation between the serum content of α_2-globulin and of TA-reacting material in their cases, while earlier workers (Perlmann, Glenn, and Kaufman, 1943 ; Chanutin and Gjessing, 1946) had reported a rise in the α-globulin fraction following experimental burns in animals. Indeed, there is some evidence that a rise of α-globulin in the serum is associated with tissue destruction, though this has recently been questioned by Marrack and Hoch (1949).

The possibility that the high TA values in our patients might be due to retention or to failure to metabolize the reacting substance cannot be excluded. The reaction mixture obtained with urine was therefore examined spectrophotometrically with a Beckman spectrophotometer with a view to measuring the excretion (if any) of TA substance. The curve differed from those obtained with serum (Fig. 3), showing that application of the reaction to urine did not provide a means of measuring the excretion of TA substance.

Another possibility that cannot be excluded is that the drugs administered, particularly penicillin, might have had some effect on the TA test. Penicillin itself did not give the colour reaction, but it is conceivable that some metabolic product might do so or that it might otherwise have some metabolic effect. However, in Case 2 the TA reaction had decreased almost to normal levels before penicillin injections had been discontinued.

Summary

A critical study was made of the serum tryptophane-perchloric acid reaction.

Results obtained with six burn cases are consistent with the view that this reaction provides a measure of tissue destruction.

I wish to thank Mr. F. A. Ibbott for making the spectrophotometric measurements (by kind permission of Dr. H. McIlwain). I am grateful also to Dr. Florence Seibert for advice; to many colleagues who provided me with clinical material, especially Dr. S. R. Elgood, Dr. G. H. Jenkins, and Mr. A. S. Aldis; to Mr. J. B. Haycraft and Dr. A. G. Watkins for permission to publish details from the patients' case-notes; to Professor J. Gough and Dr. A. Cochrane for their help.

REFERENCES

Benedict, S. R., and Franke, E. (1922). *J. biol. Chem.*, **52**, 387.
Blix, G., Tiselius, A., and Svensson, H. (1941). Ibid., **137**, 485.
Chanutin, A., and Gjessing, E. C. (1946). Ibid., **165**, 421.
Clark, E. J., Peters, R. A., and Rossiter, R. J. (1945). *Quart. J. exp. Physiol.*, **33**, 113.
Cohen, S. S. (1944). *J. biol. Chem.*, **156**, 691.
Croft, P. B., and Peters, R. A. (1945). *Lancet*, **1**, 266.
Cuthbertson, D. P. (1936). *Brit. J. Surg.*, **23**, 505.
—— McGirr, J. L., and Robertson, J. S. M. (1939). *Quart. J. exp. Physiol.*, **29**, 13.
Folin, O. (1914). *J. biol. Chem.*, **17**, 469.
Hobson, W. (1939). *Biochem. J.*, **33**, 1425.
Keyser, J. W. (1947). *Lancet*, **1**, 217.
—— (1948). *Ann. Surg.*, **127**, 605.
—— (1949). *Nature, Lond.*, **164**, 889.
King, E. J. (1946). *Micro-analysis in Medical Biochemistry*. 1st ed. London: Churchill.
—— Haslewood, G. A. D., and Delory, G. E. (1937). *Lancet*, **1**, 886.
Lustig, B., and Langer, A. (1931). *Biochem. Z.*, **242**, 320.
—— Leiner, G., and Ernst, T. (1937). *Z ges. exp. Med.*, **100**, 492.
Marrack, J. R., and Hoch, H. (1949). *J. clin. Path.*, **2**, 161.
Nilsson, I. (1937). *Biochem. Z.*, **291**, 254.
Perlmann, G. E., Glenn, W. W. L., and Kaufman, D. (1943). *J. clin. Invest.*, **22**, 627.
Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W. (1947). *J. clin. Invest.*, **26**, 90.
—— Pfaff, M. L., and Seibert, M. V. (1948). *Arch. Biochem.*, **18**, 279.
Tierney, N. A., and Peters, J. P. (1943). *J. clin. Invest.*, **22**, 599.

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EFFECT OF PARA-AMINO-SALICYLIC ACID ON PROTHROMBIN TIME

BY

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(RECEIVED FOR PUBLICATION NOVEMBER 7, 1949)

Stahmann, Huebner, and Link (1941) and Huebner and Link (1941) demonstrated by degradation reactions, which facilitated subsequent chemical synthesis, that the haemorrhagic agent $C_{19}H_{12}O_6$, isolated by Campbell from toxic sweet clover, is 3, 3′-methylene-bis (4-hydroxycoumarin). Degradation with KOH revealed that 14 of the 19 carbon atoms were recoverable as salicylic acid, $C_7H_6O_3$. The yield of salicylic acid proved to be quantitative: 7.3 mg. of the haemorrhagic agent gave 6.1 mg. of salicylic acid. Link, Overman, Sullivan, Huebner, and Scheel (1943) rationalized the possible action of dicoumarol, and were of the opinion that it must be changed chemically in the body before it acts. A schematic representation of possible degradation steps was given—viz., 3, 3′-methylene-bis (4-hydroxycoumarin) → 3-phenyl-4-hydroxycoumarin → O-hydroxyacetophenone → methyl salicylate → salicylic acid. These authors quoted unpublished work by Stahmann *et al.* to the effect that, of compounds structurally related to dicoumarol, only those show anticoagulant action which, theoretically, might yield salicylic acid or an O-hydroxybenzoic acid derivative on degradation. The effect of single doses of salicylic acid (10, 25, and 100 mg.) on the prothrombin time of rats, which had for one month been maintained on artificial low vitamin K rations, was convincingly demonstrated. The effect was quantitatively proportional to the dose, and was clearly evident within 16 hours of administration. On a dose of 300 mg. salicylic acid per day, rats (on low vitamin K diet) developed severe hypoprothrombinaemia in five days and died in 10 days with haemorrhagic manifestations resembling those of sweet clover disease in cattle. Further, it was shown that simultaneous administration of vitamin K prevented the appearance of haemorrhagic phenomena and the prolongation of the prothrombin time. Salicylic acid hypoprothrombinaemia could only be induced with difficulty in those species (e.g., rabbit and dog) in which experimental avitaminosis-K cannot readily be induced. However, the effect was greatly facilitated if the dogs' livers had previously been injured by prolonged chloroform anaesthesia. These authors concluded that, weight for weight, dicoumarol was 25 times more effective as an anticoagulant agent than salicylic acid, at the same time pointing out the rapid diffusion and elimination of the latter.

Confirmation of these experiments was obtained by Shapiro, Redish, and Campbell (1943) using human material. The prothrombin time, as determined on diluted (12½%) plasma, revealed initial prolongation between 8 and 24 hours after adequate salicylate dosage. Eight cases given 8 g. sodium salicylate daily for two consecutive days showed a prolongation of 7–12 secs.

(average 10 secs.) on diluted (12½%) plasma. Cases of cirrhosis of the liver similarly treated showed a significantly greater prolongation of prothrombin time. Acetylsalicylic acid gave substantially similar results. Vitamin K dosage (120 mg. orally and 2 mg. intramuscularly, t.d.s.) for four days, with 8 g. sodium salicylate daily, prevented the increase in prothrombin time except in a cirrhotic case which showed approximately the same increase in prothrombin time as with sodium salicylate alone. Further, it was demonstrated that the effect of dicoumarol was enhanced by sodium salicylate. Meyer and Howard (1943) also adduced evidence in confirmation of these results on human cases. With dosages of 80 gr. of aspirin daily for periods of 3 to 11 days prolongations of 2–3 seconds in prothrombin time of whole plasma were obtained. Usually the effect was discernible after one day of aspirin administration, but in most cases the maximum results were not seen until after three to four days. After cessation of the drug the prothrombin time returned to normal in two to four days. Again it was found that simultaneous administration of vitamin K obviated the effect. In view of these findings it was thought that para-amino-salicylic acid (2-hydroxy-amino-benzoic acid) might produce similar effects.

Method and Precautions

Weekly determinations of prothrombin time were carried out on cases of pulmonary tuberculosis receiving 30 g. para-amino-salicylic acid daily, and on a full and varied diet throughout.

Whole blood was collected in exactly 2 ml. amounts into tubes 3 in. × ⅜ in. each containing the residue, after evaporation at 66° C., of 0.2 ml. of a solution of ammonium oxalate (6 g.) and potassium oxalate (4 g.) in 500 ml. distilled water. Within five hours of collection prothrombin determinations in triplicate were made on plasma so obtained, using (*a*) whole plasma, and (*b*) plasma diluted to 12½% with normal saline. Controls from two members of the laboratory staff were used throughout for each weekly determination. For thromboplastin, powdered dried human brain was used (0.3 g. in 5.0 ml. normal saline incubated at 37° C. for 30 mins. with frequent mixing), 0.1 ml. being added to 0.1 ml. oxalated plasma, mixed, and placed in a water bath at 37° C. for one minute when 0.1 ml. M/40 calcium chloride was added, and simultaneously the stop-watch was started. Agitation was effected by smart rotation, but in the case of diluted plasma too rapid movement and frothing gave erroneous results. The dried brain used gave normal plasma prothrombin times of 13 to 17 seconds, depending on the sample (prepared from the same brain and stored in wax sealed 25 ml. screw-cap containers at 0–4° C.). All manipulations were carried out in calcium-free glassware. All pipettes before re-use were twice washed with normal saline in view of the observation of Link *et al.* (1943) that "a small quantity of normal plasma (or prothrombin preparation) has the capacity to restore the prothrombin time of appreciable quantities of hypoprothrombinaemic plasmas."

Concurrently with the prothrombin estimations, Kunkel (1947) tests were performed on each plasma.

Results

Results are tabulated as prolongations over parallel controls, the same two controls having been used throughout (Table I). The maximal prolongations obtained on whole plasma for individual triplicate determinations were 4, 4.5, and 6 seconds; similarly for diluted (12½%) plasma the maximal prolongations (over control times) obtained were 44.5 and 52 seconds. The Kunkel test in all except four cases, who had very severe and extensive disease, showed no significant deviation from normal.

TABLE I

RESULTS OF PROTHROMBIN ESTIMATIONS AND KUNKEL TESTS ON PLASMA

Patient	Mean Deviation of Prothrombin Time from Controls				Kunkel Tests	
	Whole Plasma (Sec.)	No. of Triplicate Determinations	Diluted Plasma (Sec.)	No. of Triplicate Determinations	Mean Deviation from Control (Units)	No. of Tests
1	+0.85	14	+5.17	12	+2.60	14
2	+1.44	14	+10.16	12	+2.80	14
3	+1.75	13	+12.38	11	+0.03	13
4	+1.57	15	+6.80	13	−0.81	15
5	+1.80	14	+12.56	12	+3.10	14
6	+2.07	14	+10.90	12	+2.00	14
7	+1.30	15	+8.06	13	−1.33	15
8	+1.34	13	+5.80	11	−1.90	13
9	+0.43	15	+1.80	13	+8.80	15
10	−0.54	4	−6.15	2	+0.97	4
11	+1.95	10	+9.95	10	+0.90	11
12	+1.47	15	+10.40	13	+1.10	15
13	+4.77	6	+1.10	4	+3.66	6
14	+0.76	12	+0.07	10	+0.00	12
15	+2.78	14	+8.10	12	+5.49	14
16	+3.85	2	Not done		+2.80	3
17	+1.96	12	+15.40	12	+0.25	12
18	+2.14	9	+13.20	9	+0.30	9
19	+2.70	5	+11.40	5	+5.56	5
20	+1.75	4	+6.90	4	+1.05	4
21	+1.66	3	+9.80	3	+2.90	3
22	+0.00	2	+0.50	2	−0.70	3
23	+0.25	8	+1.80	8	−0.90	8
24	+2.30	7	+16.50	7	−1.46	7
25	+0.56	9	+4.90	9	+3.70	9
26	+2.25	6	+5.75	6	+8.10	6
27	+2.25	4	+11.80	4	+2.60	4
28	+0.08	6	+0.90	6	+0.75	6
29	+2.00	3	+3.2	3	+1.45	2
Mean value	+1.6	266	+8.0	238	+1.8	270
Controls mean	16.3	15	42.8	13	8.63	15

Discussion

The clinical significance of the prolongation of prothrombin time by P.A.S. would appear to be slight or even negligible for otherwise normal cases on full diet. The maximum prolongation obtained on whole plasma in this series—viz., 6 seconds—gave a prothrombin time of 22 seconds which, on a composite dilution curve for six normal plasmas, corresponds to 31.5% prothrombin. Spontaneous haemorrhages do not, in my experience, occur with prothrombin concentrations in excess of 10% to 15%. However, the dangers of such an occurrence must be regarded as increased where concomitant liver disease—e.g., cirrhosis or amyloid—exists. This prothrombinopenic effect of P.A.S. does not appear to be cumulative, as this was not found in patients receiving 30 g. per day for as long as three and four months. Indeed, it has been a constant feature that the small elevation of the prothrombin

time, which occurs within a few days to a week of inception of P.A.S. therapy, is maintained, with minor fluctuations, throughout.

The hypothesis of Link *et al.*, that dicoumarol may act by degradation *in vivo* to salicylates, does not appear to be borne out by these results. If this were true one would expect much greater prolongations of prothrombin times on such high doses as 30 g. P.A.S. per day, and on the doses of salicylates used by other investigators. Some, but only very slight, action of dicoumarol may be due to salicylate products of decomposition.

From the serial Kunkel tests, it may be said that γ globulins and, indirectly, liver function were substantially unaffected by the therapy. This and the prothrombin findings accord with those of Lehmann (verbal communication).

The prophylactic and therapeutic effects of vitamin K are well shown in the accompanying graph (Fig. 1). The significance of this as regards diet needs no comment.

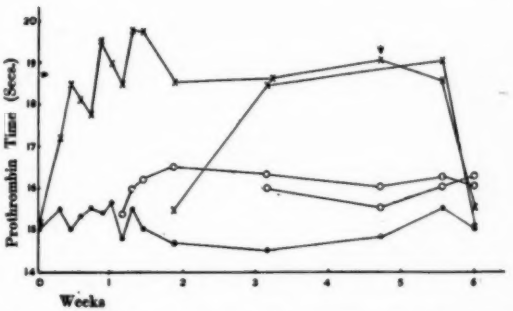

FIG. 1.—The relation between the prothrombin time of whole plasma (secs.) and time (weeks) of four tuberculous patients given 30 g. P.A.S. daily. A normal control (●) is included. The prophylactic effect (O) and the therapeutic effect (×) of 10 mg. daily oral doses of synthetic vitamin K is shown.

It will be seen from Table I that, while there is a good measure of agreement between values obtained on whole and diluted plasmas, some discrepancies (e.g., in Case No. 13) do occur. Obviously the factor of dilution of unknown quantities or substances occurs, as well as errors inherent in dilution of plasma. Apart from this, the end-point of clotting of recalcified diluted plasma is not always clear, and experience and meticulous adherence to a rigid technique is necessary if results are to be accurate and reproducible.

Summary

The effect of P.A.S. on the prothrombin time was investigated in a number of patients receiving large therapeutic doses over long periods for the treatment of pulmonary tuberculosis. A slight prolongation was noted, but this is thought to be without clinical significance in patients receiving a full diet.

Grateful acknowledgment is made to Dr. D. G. Madigan, under whose care these patients were, for his generous co-operation and encouragement in this study.

REFERENCES

Huebner, C. F., and Link, K. P. (1941). *J. biol. Chem.*, **138**, 529.
Kunkel, H. G. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 217.
Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D. (1943). *J. biol. Chem.*, **147**, 463.
Meyer, O. O., and Howard, B. (1943). *Proc. Soc. exp. Biol., N.Y.*, **53**, 234.
Shapiro, S., Redish, M. H., and Campbell, H. A. (1943). Ibid., **53**, 251.
Stahmann, M. A., Huebner, C. F., and Link, K. P. (1941). *J. biol. Chem.*, **138**, 513.

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DRIED DISC TECHNIQUE FOR DETERMINING SENSITIVITY TO THE ANTIBIOTICS

BY

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Since the introduction of the antibiotics, the disc technique has been used by various workers for their assay (Vincent and Vincent, 1944 ; Epstein, Foley, Perrine, and Lee, 1944 ; Heatley, 1944), and also for the detection of bacterial sensitivities (Morley, 1945).

An attempt has been made to standardize the method of preparing and storing the disc impregnated with antibiotic to determine bacterial sensitivity within 18 hours' incubation. To accomplish this the discs must give a constant standard zone of growth inhibition when tested with a known sensitive organism, be conveniently prepared and dried, and have a sufficiently high concentration of the antibiotic impregnated into them to remain stable when stored.

Each disc is a standard size and is impregnated with a standard volume and concentration of the antibiotic. It was found, after experimenting with various-sized discs, that a 9 mm. disc cut from " No. 633 Hayle Mills " blotting-paper would absorb approximately the whole volume of one drop (0.02 ml.) from a 50-dropping pipette. As the discs are dried after impregnation by leaving them in an open incubator for two hours, it was found necessary to use a much higher concentration of antibiotic than has been used by previous workers (Morley, 1945). The concentration ultimately used gave a zone sufficiently large for measurement, and, at the same time, without a tendency to inhibit completely the growth of the pool inoculum.

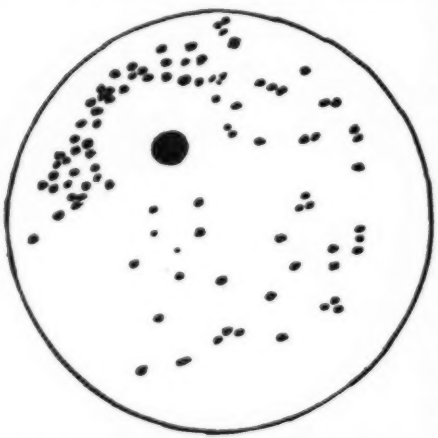

FIG. 1.—Diagram showing disc in optimum position to prevent complete inhibition of growth when inoculum is small.

Neither the size of the inoculum nor the degree of moisture on the plates seems to have an appreciable effect on the zoning. It has been found necessary to place the discs in an optimum position (Fig. 1), first, to avoid completely inhibiting the growth of

inoculum and, secondly, to show the sensitivity of isolated colonies. The disc should not be less than 15 mm. from the edge of the plate, as there appears to be a reflection wave effect from the edge of the plate which gives inaccurate zoning. The discs cannot be used on penicillinase plates ; in cases treated with penicillin a primary isolation of bacteria has to be made before discs can be used for testing their sensitivity.

Dried Penicillin Discs

Method of Preparation.—Using "No. 633 Hayle Mills" blotting-paper, discs are cut out with a 9 mm. cork borer, and are then separated out in a large size petri dish and sterilized in a hot air oven. Each disc is then impregnated with 1 drop (0.02 ml.) of a freshly made solution of 1,000 units penicillin per ml. using a 50-dropper pipette. The discs are dried in an incubator for two hours with the lid of the petri dish slightly open. Any moisture forming in the lid of the petri dish is removed with sterile filter paper and the discs are then stored in the refrigerator. They are stable for six to eight months.

Sensitivity Interpretation.—Measurements are made from the edge of the disc to the line of growth.

Examples.—*Staph. Mayo* gives 15 mm. = 0.025 unit per ml. tube sensitivity ∴ sensitive organism

Staph. 25W (Watford strain) gives 8 mm. = 0.25 unit per ml. tube sensitivity ∴ moderately sensitive organism.

Staph. 16X (Watford strain) gives 3 mm. = 0.3 unit per ml. tube sensitivity ∴ slightly sensitive organism.

Staph. 19BY (Watford strain) gives nil = 0.5 unit per ml. tube sensitivity ∴ resistant organism.

Dried Streptomycin Discs

Method of Preparation.—Discs are prepared and sterilized as for penicillin, and are then impregnated with 10,000 μg. per ml. streptomycin and dried as before. The discs are stable for about six months.

Sensitivity Interpretation.—Measurements are made from the edge of the disc to the line of growth.

Examples.—*Bact. coli* (Watford strain) gives 12 mm. = 10–5 μg. per ml. tube sensitivity ∴ sensitive organism.

Micrococcus 14W (Watford strain) gives no zone = 200 μg. per ml. tube sensitivity ∴ resistant organism.

Dried Aureomycin Discs

Method of Preparation.—Discs are prepared and sterilized as for penicillin and are then impregnated with a 2,500 μg. per ml. solution of aureomycin, and dried as before. The 2,500 μg. per ml. solution of aureomycin was prepared by dissolving the hydrochloride salt in sterile distilled water, buffered with acetate at *p*H 5. Owing to the instability of aureomycin in solution, reliable methods for tube sensitivity testing were not found, but although the values given are not absolute, they are at least relative, and in point of fact do correspond respectively with the zoning. These discs to date have been stable for six months.

Sensitivity Interpretation.—Measurements are made from the edge of the disc to the line of growth.

Examples.—*Staph. Mayo* gives 12 mm. = 0.25 μg. per ml. tube sensitivity ∴ sensitive organism.
Bact. coli (Watford strain) gives 3 mm. = 2.5 μg. per ml. tube sensitivity ∴ moderately sensitive organism.
Proteus (Watford strain) gives nil = 20 μg. per ml. tube sensitivity ∴ resistant organism.
Staph. 19BY (Watford strain) gives 12 mm. = 0.25 μg. per ml. tube sensitivity ∴ a sensitive penicillinase-producing Staph.

Dried Chloromycetin Discs

Method of Preparation.—Discs are prepared and sterilized as for penicillin and are then impregnated with a solution of 2,500 μg. per ml. of chloromycetin and dried as before. To date the discs have been stable for two months.

Sensitivity Interpretation.—Measurements are made from the edge of the disc to the line of growth.

Examples.—*Staph. Mayo* gives 11 mm. = 3.1 μg. per ml. tube sensitivity ∴ sensitive organism.
Bact. coli (Watford strain) gives 9 mm. = 6.2 μg. per ml. tube sensitivity ∴ moderately sensitive organism.
Proteus (Watford strain) gives 8 mm. = 12.5 μg. per ml. tube sensitivity ∴ slightly sensitive organism.

Table I analyses quantitatively certain bacterial sensitivities to penicillin, streptomycin, aureomycin, and chloromycetin.

TABLE I

AN APPROXIMATE QUANTITATIVE EVALUATION OF BACTERIAL SENSITIVITY TO ANTIBIOTICS

Antibiotic	Concentration in Disc	Test Organism	Zoning (mm.)	Corresponding Tube Sensitivity	Sensitivity
Penicillin	1,000 units per ml.	*Staph. Mayo*	15	0.025 unit per ml.	Sensitive
		Staph. 25W	8	0.25 ,, ,,	Moderately sensitive
		Staph. 16X	3	0.3 ,, ,,	Slightly sensitive
		Staph. 19BY	0	0.5 ,, ,,	Resistant
Streptomycin	10,000 μg. per ml.	*Bact. coli*	12	10–5 μg. per ml.	Sensitive
		Micrococcus 14W	0	200 ,, ,,	Resistant
Aureomycin	2,500 μg. per ml.	*Staph. Mayo*	12	0.25 ,, ,,	Sensitive
		Bact. coli	3	2.5 ,, ,,	Moderately sensitive
		Proteus	0	20 ,, ,,	Resistant
		Staph. 19BY (penicillinase-producer)	12	0.25 ,, ,,	Sensitive
Chloromycetin	2,500 μg. per ml.	*Staph. Mayo*	11	3.1 ,, ,,	,,
		Bact. coli	9	6.2 ,, ,,	Moderately sensitive
		Proteus	8	12.5 ,, ,,	Slightly sensitive

Summary

A description is given of a method of determining the sensitivities of bacteria to the antibiotics by means of impregnated and dried blotting-paper discs. The method aims at standardizing the interpretation of this disc method and is for use in a routine laboratory, the discs being applied to the plates that have been sown direct with the material under investigation.

A zone of inhibition is given which is related to the concentration of the antibiotic necessary to inhibit growth. *Measurement of the zones is a fairly accurate guide to the sensitivity of the organism*, and therefore to the adequacy of the antibiotic dosage.

The discs are easily prepared and dried, convenient to handle, being immediately ready for use, and can be stored without loss of potency within a known period of time.

I should like to thank Drs. Stokes and Schwabacher, in whose laboratories this work was originally done. I should also like to thank Dr. Jones for his assistance.

REFERENCES

Epstein, J. A., Foley, E. J., Perrine, Isabel, and Lee, S. W. (1944). *J. Lab. clin. Med.*, **29**, 319.
Heatley, N. G. (1944). *Biochem. J.*, **38**, 61.
Morley, D. C. (1945). *J. Path. Bact.*, **57**, 379.
Vincent, J. J., and Vincent, H. W. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 162.

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HYPOPITUITARISM IN A MALE DUE TO GIANT CELL GRANULOMA OF THE ANTERIOR PITUITARY

BY

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(RECEIVED FOR PUBLICATION FEBRUARY 15, 1950)

It has been emphasized by Sheehan (1948) that the criterion for the diagnosis of hypopituitarism is destruction of the anterior pituitary, the most frequent cause being post-partum necrosis. Hypopituitarism in the male is relatively uncommon. Escamilla and Lisser (1942), in a survey of 101 "typical clinical cases with pathological verification," found only 35 in the male.

The following case, which was fully investigated clinically, is reported because of the unusual pathological lesion in the pituitary.

Case Report

The patient, a man aged 29 years, was admitted to the Manchester Royal Infirmary under the care of Dr. C. S. D. Don on August 3, 1948. He had been under the care of Dr. F. Janus at the Manchester Royal Eye Hospital, on account of blurring of vision and pain in the left eye. These symptoms were of three months' duration and the diagnosis made was interstitial keratitis and iritis of the left eye. In addition there were vitreous opacities and an old anterior choroiditis in the right eye.

He also complained of weakness during the past two years; this was rapidly becoming more severe so that he was unable to carry on any occupation. On close questioning it was further elicited that during this period he had noted an increasing sensitivity to cold and loss of libido, with infrequent erections and no emissions in the last 18 months. Pubic and axillary hair had been lost. More recently he had had anorexia and vomiting, and had lost 6 lb. in weight. For six months there had been occasional transient attacks of sweating, but no accompanying disturbance of consciousness.

The patient was married at 23, and had a healthy child aged 5 years. A second child, two years later, died at 10 days from "lung trouble." The patient's mother, aged 69, was blind and demented. His two brothers and two sisters were healthy.

Clinical Examination.—On admission he was thin, but normally proportioned (height, 62 in., weight, 114 lb.). His intelligence was moderate; speech, comprehension, and movement were all rather slow. The skin was smooth with a peculiar waxy pallor, but the mucous membranes were of normal colour. There was no abnormal pigmentation. The hair was dark and plentiful on the head and eyebrows,

very scanty in the pubic region, and absent in both axillae. Body hair was almost completely absent, and although the patient had not shaved for a week there was little beard.

The left eye showed a ground-glass opacity of the cornea (Fig. 1), with a blepharo-conjunctivitis. The left upper central incisor tooth was barrel-shaped, with notching and narrowing at the cutting edge (Fig. 2). The rest of the teeth were normal apart from dental caries. There was no deafness.

FIG. 1.—Left eye showing ground glass opacity of cornea.

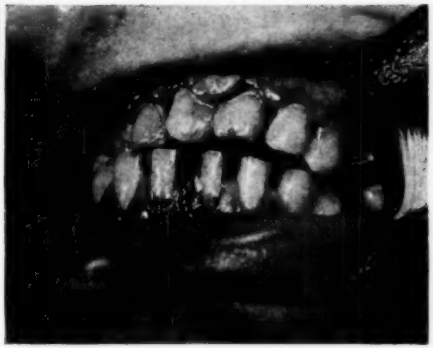


FIG. 2.—Barrel-shaped left upper central incisor, with narrowing and notching of cutting edge.

No abnormal signs were found in the chest, abdomen, and nervous system. The pulse rate was 62, and the blood pressure 95/75 mm. Hg. The penis and testes were small.

Laboratory Investigations.—A blood count showed : red cells, 4,380,000/mm. ; haemoglobin 11.6 g./100 ml. ; colour index 0.95 ; white cells 9,400/c.mm. (7.5% eosinophils).

Blood Wassermann and Kahn reactions: negative.

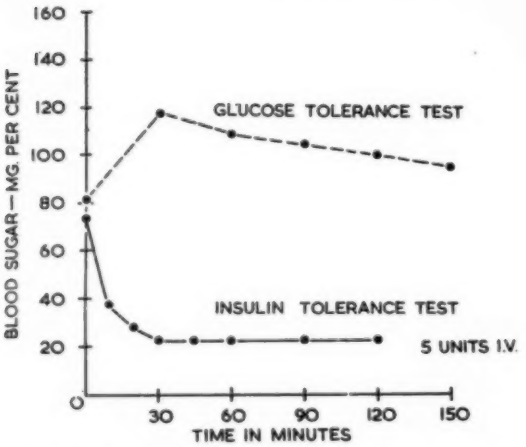
Cerebrospinal fluid: 8 lymphocytes/c.mm. ; protein 50 mg. % ; slight globulin opalescence ; Lange curve normal ; Wassermann negative.

Mantoux reaction: positive.

Basal metabolic rate: −30%.

X-ray examination of the skull, chest, and epiphyses, all normal.

Kepler test: positive.
- Volume of night urine: 134 ml.
- Maximum hourly specimen: 31 ml.
- Plasma chloride: 491 mg.%
- Blood urea: 20 mg.%
- Urinary chloride: 538 mg.%
- Urinary urea: 2.2 g.%
- Mayo Clinic factor: 23



Excretion of 17-ketosteroids: 6.1 mg. and 2.6 mg./day.

Excretion of gonadotrophins: 26 and 14 mouse units/day (Dr. A. M. Hain).

Glucose tolerance and insulin tolerance curves as in Fig. 3.

FIG. 3.—Glucose and insulin tolerance curves.

Blood chemistry:

Calcium	10.5 mg.%	Globulin	2.5 g.%
Inorganic phosphorus	3.8 mg.%	Alkaline phosphatase	8.3 units 100 ml.
Cholesterol	260 mg.%		
Sodium	335 mg.%	Thymol turbidity	7.8 units 100 ml.
Potassium	19.2 mg.%		
Albumin	3.3 g.%		

Progress.—The patient developed attacks of drowsiness, alternating with delusions of persecution and hallucinations. There was no excessive sweating, but a blood sugar estimation during an attack was only 33 mg.% Intravenous glucose raised the blood sugar level but had only a temporary effect on the clinical picture. Adrenal cortical extracts, salt, and methyl testosterone were also given, with little response. He died on October 18, 1948.

Pathological and Histological Examination

The body was that of a well-nourished adult male, with almost complete loss of pubic and axillary hair. The only other abnormal naked eye findings, apart from an early bronchopneumonia, were related to the endocrine glands and reproductive organs. The meninges covering the sella-turcica were thickened and adherent, and the pituitary was flattened with a depression on the upper surface. The pituitary stalk was elongated and the tuber cinereum prominent. The adrenals and testes were small. There was no appreciable enlargement of lymphoid tissue.

Pituitary.—The capsule was thickened, showing dense fibrosis with focal infiltration of lymphocytes and plasma cells. There was complete disorganization of the anterior pituitary (Fig. 4), so that only isolated parenchymal cells could be identified. Scattered throughout were granulomatous foci resembling tubercles, consisting of groups of epithelioid cells with surrounding infiltration by lymphocytes and plasma cells. There was no caseation, and some granulomatous foci were healing by fibrosis and hyalinization. There were numerous large, multinucleated, Langhans' giant cells, both in the granulomatous foci and in the cellular infiltration (Fig. 5). Many of these giant cells were much larger than the type seen in tuberculosis, and resembled foreign body

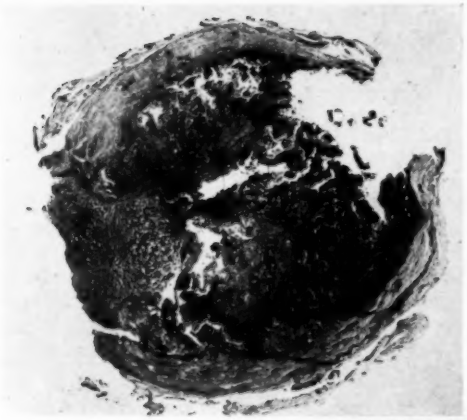


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FIG. 4.—Pituitary showing capsular fibrosis and disorganization of anterior lobe. (Haematoxylin and eosin × 6.)

FIG. 5.—Granulomatous lesions in pituitary, with lymphocytic infiltration and Langhans's giant cells. (Haematoxylin and eosin × 100.)

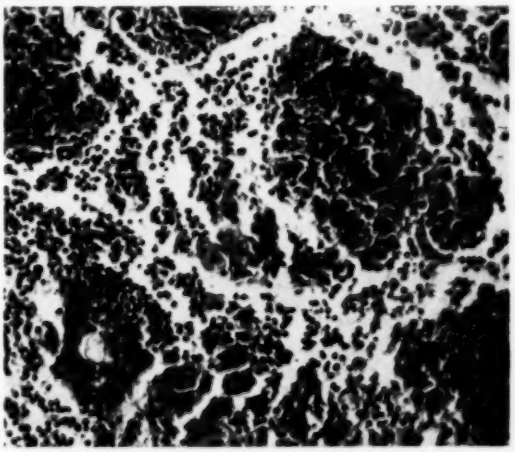

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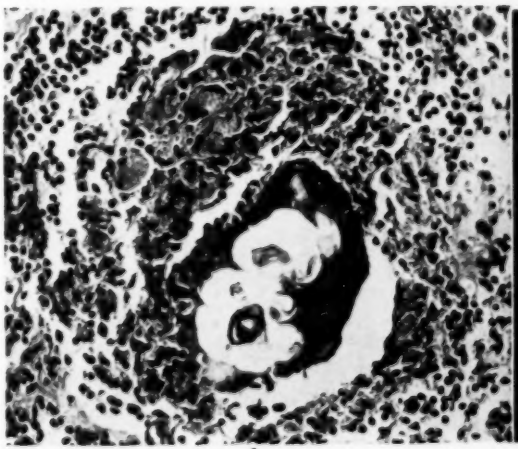
FIG. 6.—Massive vacuolated giant cell with basophilic inclusions in granulomatous lesion. (Haematoxylin and eosin × 270.)

FIG. 7.—Large vacuole in giant cell filled by refractile crystalline material. (Polarized light. Safranin × 270.)



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giant cells. Many showed vacuolation and contained fibrillary basophilic material (Fig. 6). In unstained sections, and in sections which had not been treated with acid alcohol, these inclusions were seen, using polarized light, to be embedded in a refractile crystalline mass (Fig. 7). This accounted for the irregular staining of the fibrillary material. Micro-incineration showed that the crystalline material was inorganic, and microchemical tests indicated it to be chiefly calcium. The vacuolation of the giant cells in sections stained by haematoxylin and eosin was thus apparently due partly to the solubility of the crystalline material in acid alcohol, and partly to the material dropping from the section. Many of these giant cells appeared to be formed by the fusion of smaller giant cells. Basophil fibrillary fragments were also seen lying free in the centre of granulomatous foci and in the disorganized gland. Sections stained for spirochaetes and tubercle bacilli were negative. The posterior pituitary was normal except for a few small lymphocytic foci.

Adrenals.—There was marked hypoplasia of the cortex involving all the zones, but there was no fibrous zone replacing the zona reticularis, as noted by Sheehan and Summers (1949) in severe hypopituitarism. The cells showed a normal fat content. Multiple small granulomatous lesions resembling those seen in the pituitary were present in both the cortex and medulla. Small Langhans' giant cells were present, but no massive giant cells with inclusions.

Thyroid.—There were atrophic changes with small acini, areas of fibrosis, and lymphocytic infiltration.

Testes.—There was widespread atrophy of the epithelium of the seminiferous tubules with complete absence of spermatogenesis. The tubules showed hyaline thickening of the basement membrane, and there was a fine intertubular fibrosis with scattered lymphocytes. No interstitial cells could be identified. Numerous thin-walled veins, especially in the rete testis, showed granulomatous foci in the walls just beneath the endothelium. These closely resembled the lesions in the pituitary. A few small Langhans' giant cells were present. Many other vessels showed focal fibrosis suggesting similar healed lesions.

Skin.—The axillary skin showed atrophy of the epithelium and rete pegs, elastosis in the superficial dermis, only an occasional atrophic hair follicle, and complete absence of sebaceous glands. Groups of sweat glands were present, with foci of lymphocytes around the vessels.

Apart from scanty lymphocytic infiltration in the portal tracts in the liver, no changes were noted in the heart, pancreas, spleen, lymph glands, or hypothalamus.

Discussion

The clinical picture, with marked asthenia, sensitiveness to cold, decrease in libido, loss of pubic and axillary hair, and attacks of hypoglycaemia, is quite characteristic of hypopituitarism. The results of the biochemical investigations support the diagnosis, and will now be briefly discussed, since they are the means of assessing the functional activity of the endocrines. Diminished thyroid activity is indicated by the low basal metabolic rate. The Kepler test, insulin tolerance test, and the excretion of 17-ketosteroids afford some indication of the activity of the adrenal cortex. The positive Kepler test (Robinson, Power, and Kepler, 1941) shows deficiency of the electrolyte regulating hormones of the adrenal cortex, while the marked "hypoglycaemia unresponsiveness" in the insulin tolerance test (Fraser and Smith, 1941) indicates a lack of oxycorticosteroids (and possibly lack of the diabetogenic hormone of the anterior pituitary). The 17-ketosteroid excretion was reduced, but

higher than that found by most workers (Fraser and Smith, 1941 ; Fraser *et al.*, 1941). Simpson (1948) states that he has had one definite case of hypopituitarism with an excretion of 12 mg./day. The gonadotrophin output, as a direct indication of the activity of the anterior pituitary, was within normal limits, in contrast to the low excretion recorded by Klinefelter, Albright, and Griswold (1943). These values indicate biochemically that the hypopituitarism was not severe and was probably of recent onset. Pathologically, the degree of atrophy of the thyroid, adrenal, and testis is not of the advanced type that occurs in long-standing hypopituitarism, and accords with the subacute nature of the pituitary lesion. The extreme disorganization of the anterior pituitary is, however, difficult to reconcile with the normal gonadotrophin output.

Clinically the cause of the pituitary damage was thought to be congenital syphilis. This diagnosis entirely depended on the condition of the eyes and teeth. The ophthalmologist considered the interstitial keratitis to be diagnostic of congenital syphilis. The dental surgeon (Mr. Matheson) stated that the appearance of the left upper central incisor indicated a developmental defect seen in congenital syphilis. However, the Wassermann and Kahn reactions of the blood and cerebrospinal fluid were negative. Congenital syphilis was not established in any of the siblings, and the Wassermann reaction of the mother was negative.

The characteristic features of the pituitary lesion were the multiple granulomatous nodules resembling tubercles, the widespread lymphocytic and plasma cell infiltration, and the many large vacuolated Langhans' giant cells containing basophilic inclusions. This appearance does not suggest a syphilitic process. Lesions of the pituitary caused by syphilis vary from gummatous necrosis (Simmonds, 1914) to diffuse interstitial fibrosis (Good and Newman, 1929 ; Hirsch, 1922 ; Farquharson and Graham, 1931), and are usually associated with gummatous necrosis in other organs (Sheehan and Summers, 1949).

The pituitary lesion in our case closely resembles that described by Simmonds (1918) in five elderly females in a series of 2,000 necropsies. These patients died from various types of acute and chronic diseases, but there were no symptoms referable to pituitary damage. The lesion was limited to the anterior pituitary, and not found in other organs. Simmonds considered that the process was neither tuberculous nor syphilitic but was a condition *sui generis*. Sheehan and Summers (1949) have classified a number of similar cases as giant cell granuloma of the anterior pituitary, both in subacute (17 cases) and healed (9 cases) stages. Most of the cases occurred in females between the ages of 45 and 65 years. Six of the subacute cases showed similar lesions in the brain and meninges, but otherwise the process appeared localized to the anterior pituitary. They concluded that most cases appeared to be examples of a specific process of unknown aetiology, but a few were said to be syphilitic. These, however, showed little or no evidence of syphilis in other organs. We have reviewed many of these cases of so-called giant cell granuloma, and though several closely resemble our case the evidence for the inclusion of others is rather doubtful.

The histological features of giant cell granuloma are in many ways similar to those of sarcoid. Tillgren (1935) described a male aged 42 years, with typical sarcoid in lymph glands, skin, and bone, who developed diabetes insipidus. The pituitary

lesion, showing large Langhans' giant cells, with basophilic inclusions, closely resembles our case. Similar features were seen in a female aged 41 years, reported by Kraus (1942). Barber (1945) reported the development of hypopituitarism in a male aged 28 years, with typical clinical evidence of sarcoidosis. Longcope (1941) has remarked that giant cells are not regularly present in sarcoid but are quite common, and are often large and irregular and contain peculiar basophilic inclusions, sometimes suggesting calcified material. These inclusions have been considered by many observers to be characteristic of sarcoidosis. We have seen inclusions, closely resembling those in our case of giant cell granuloma, in a typical case of sarcoidosis which had been followed over a period of 12 years. Schaumann (1941), however, stated that Metchnikoff had observed this type of inclusion body in an ape infected with tuberculosis. Professor Baker has kindly shown us sections of an inguinal node from a girl aged 3 years, who, clinically and radiologically, had tuberculous disease of the right hip joint. This lymph node showed scattered granulomatous tubercles with several large giant cells containing similar refractile basophilic inclusions. While this similarity to sarcoid should be considered, we have no convincing evidence that giant cell granuloma and sarcoid are the same process. Other lesions characteristic of sarcoid are absent in our case. The occurrence of granulomatous lesions in the testis and adrenal does, however, suggest the possibility of a systemic infection, though it is striking that these lesions have only occurred in the adrenal and testis, both of which are subject to a trophic hormone deficiency.

In the recent literature a similar type of lesion was reported by Coleman and Meredith (1940). In a patient with symptoms suggesting a pituitary neoplasm, a mass of tissue was removed surgically, and this histologically resembled hyperplastic tuberculosis or sarcoidosis. Giant cells were present but no inclusions were mentioned. This patient was alive and apparently well three and a half years later, suggesting that the lesion may have been limited to the pituitary region. Glass and Davis (1944) reported a granuloma of the pituitary, associated with panhypopituitarism, in a male aged 54 years. The lungs showed an infiltrative lesion with areas of caseation, and the anterior pituitary was largely replaced by dense scar tissue. The photographs are not helpful in assessing the aetiology, but it seems reasonable to relate the pituitary and lung lesions which appear to be due to either tuberculosis or sarcoid. Letchworth (1924) described a male, aged 16 years, with diabetes insipidus. There was a mass, surrounding the pituitary and optic chiasma, which was described as " tuberculous " but with no evidence of tubercle elsewhere. No histological details are given.

Sheehan and Summers (1949) described the formation of these large giant cells in two cases of " healed giant cell granuloma " and noted the development of central foaminess, followed by vacuolation and finally a clear space sometimes containing basophilic fibrillary inclusions. We consider that in our case the vacuolation in the giant cells was largely an artefact due to the solubility in acid alcohol of the crystalline material enveloping the fibrillary inclusions. This basophilic material has been seen lying free in granulomatous areas and its staining reactions and appearance suggest an origin from the reticular framework of the gland. These fragments are then probably engulfed by giant cells.

Sheehan and Summers (1949) commented on the possibility that the epithelioid and giant cells of the granulomatous foci might be derived from parenchymal cells, the lesion being analogous to the pseudogranulomatous nodules of the atrophying thyroid. This suggestion is hardly tenable in our case since similar granulomatous lesions were found in the adrenal and testis.

In conclusion our case is best considered as belonging to the group of giant cell granulomas of the anterior pituitary of unknown aetiology. Whether this is a specific disease process cannot be decided on the present evidence. Our case is of interest since it clearly shows that the process is not strictly localized to the anterior pituitary.

Summary

A case of hypopituitarism in a male with clinical evidence of congenital syphilis is recorded.

The lesion in the anterior pituitary was a giant cell granuloma.

The aetiology of the condition has been discussed.

The presence of additional lesions in the adrenal and testis suggests the possibility of a systemic infection.

We wish to thank Dr. C. S. D. Don and Dr. F. Janus for permission to publish this case, Professor H. L. Sheehan for his invaluable opinion, and Professor S. L. Baker for helpful advice and criticism. Our thanks are also due to Mr. H. Varley, M.Sc., for the biochemical investigations, Dr. A. M. Hain for the gonadotrophin estimations, Mr. J. Kilshaw (Department of Medical Photography) for the photographs, and Mr. F. Ward for the photomicrographs.

REFERENCES

Barber, H. W. (1945). *Proc. R. Soc. Med.*, **39**, 92.
Coleman, C. C., and Meredith, J. M. (1940). *Arch. Neurol. Psychiat., Chicago*, **44**, 1076.
Escamilla, R. F., and Lisser, H. (1942). *J. clin. Endocrinol.*, **2**, 65.
Farquharson, R. F., and Graham, D. (1931). *Trans. Ass. Amer. Phys.*, **46**, 150.
Fraser, R. W., Forbes, A. P., Albright, F., Sulkowitch, H., and Reifenstein, E. C. (1941). *J. clin. Endocrinol.*, **1**, 234.
—— and Smith, P. H. (1941). *Quart. J. Med.*, n.s., **10**, 297.
Glass, S. J., and Davis, D. S. (1944). *J. clin. Endocrinol.*, **4**, 489; 611.
Good, T. S., and Newman, K. O. (1929). *Lancet*, **1**, 765.
Hirsch, S. (1922). *Dtsch. Arch. klin. Med.*, **140**, 323.
Klinefelter, H. F., Albright, F., and Griswold, G. C. (1943). *J. clin. Endocrinol.*, **3**, 529.
Kraus, E. J. (1942). *J. Lab. clin. Med.*, **28**, 140.
Letchworth, T. W. (1924). *Brit. med. J.*, **1**, 1127.
Longcope, W. T. (1941). *J. Amer. med. Ass.*, **117**, 1321.
Robinson, F. J., Power, M. H., and Kepler, E. J. (1941). *Proc. Mayo Clin.*, **16**, 577.
Schaumann, J. (1941). *Acta med. scand.*, **106**, 239.
Sheehan, H. L. (1948). *Proc. R. Soc. Med.*, **41**, 187.
—— and Summers, V. K. (1949). *Quart. J. Med.*, n.s., **18**, 319.
Simmonds, M. (1914) quoted by Jaffé, R. (1922). *Frankfurt. Z. Path.*, **27**, 324.
—— (1918). *Virchows Arch.*, **223**, 281.
Simpson, S. L. (1948). *Proc. R. Soc. Med.*, **41**, 192.
Tillgren, J. (1935). *Brit. J. Derm. Syph.*, **47**, 223.

J. clin. Path. (1950), 3, 130.

THE CONSUMPTION OF PROTHROMBIN DURING COAGULATION

THE DEFECT IN HAEMOPHILIA AND THROMBOCYTOPENIC PURPURA

BY

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During the process of normal blood coagulation prothrombin is converted into thrombin. As a result serum contains considerably less prothrombin than the plasma from which it is derived, the prothrombin having been consumed during the coagulation of the blood. In certain conditions this conversion is incomplete and much prothrombin remains in the serum. Often this is not obvious from the usual tests of clotting function since only a small amount of thrombin is necessary for apparently normal coagulation of blood in glass tubes. This defect is unmasked by the prothrombin consumption test which offers another method for studying the efficacy of the blood clotting process.

Bordet and Delange (1912) were the first to observe deficient prothrombin consumption in normal platelet-free plasma, and their findings were confirmed and elaborated by Quick (1947) and Quick, Shanberge, and Stefanini (1949a). A similar defect was reported in thrombocytopenic purpura (Soulier, 1948 ; Quick *et al.*, 1949b, and Alexander and de Vries, 1949a), while at the same time Soulier (1948) pointed out that there were a number of haemorrhagic syndromes where, although the platelets were not numerically decreased, there was defective prothrombin consumption, and he postulated a qualitative platelet defect in these conditions. Brinkhous (1939) had pointed out a prothrombin consumption defect in haemophilia, and this observation was confirmed and extended by Quick (1947), Soulier (1948), and Alexander and de Vries (1949b). The observations of Quick (1947) and Soulier (1948) showed that thrombocytopenic plasma and haemophilic plasma consumed little prothrombin during coagulation. On mixing and recalcifying these two types of plasma, a normal amount of prothrombin was used up in the clotting process. It was therefore argued that the defective consumption of prothrombin was due to a different deficiency in the two types of plasma, since each appeared to contain an excess of a factor lacking in the other. It is the purpose of this study to confirm and extend these observations.

Technique

Reagents were prepared as follows.

Brain Thromboplastin.—Fresh human brain was collected from the post-mortem room, all superficial vessels and meninges removed, and the substance ground with

* Cecil John Adams travelling scholar.

acetone in a mortar. The acetone was poured off and replaced several times and the remaining granular powder dried on a suction filter. When completely free of acetone the powder was kept in bottles at room temperature. This material can be stored for three months without deterioration.

When required for the test 0.5 g. dried brain was mixed with 5 ml. normal (0.85%) saline and incubated for 15 minutes at 37° C. It was then centrifuged at 500 revolutions per minute for one minute and the supernatant emulsion diluted with normal saline to the concentration which had been shown by trial for that particular batch to give the best results. This was usually a 1/5 dilution. A fresh suspension of brain was made daily.

Calcium Chloride Solution.—This was made up to a strength of $\frac{\text{molar.}}{40}$

Fibrinogen.—Jaques' (1943) method was followed. A 3-mol. phosphate buffer at *p*H 6.6 was made by dissolving 817 g. of anhydrous KH_2PO_4 in 1,000 ml. of distilled water. After warming, the volume was made up to 2,000 ml. and the solution filtered: 2 mol., 1 mol., or $\frac{\text{mol.}}{4}$ solutions were made from this stock solution.

Stored citrated human plasma was used after preliminary adsorption with an excess of barium sulphate. To 100 ml. of adsorbed plasma 100 ml. of 2 mol. phosphate buffer is added. After centrifuging, the precipitate is washed with 100 ml. of mol. phosphate buffer and then dissolved in 75 ml. of $\frac{\text{mol.}}{4}$ phosphate buffer. This precipitation with an equal volume of 2 mol. phosphate buffer, washing with mol. phosphate buffer and redissolving the precipitate in three-quarters of the volume of $\frac{\text{mol.}}{4}$ phosphate buffer, was repeated twice. The final precipitate was dissolved in 50 ml. of normal (0.85%) saline containing 0.16% sodium citrate. The solution was dialysed for 24 hours against 3% sodium citrate in normal saline, the dialysing fluid being changed after the first 12 hours. Excess solids were finally removed by centrifugation. The fibrinogen was stored at −20° C. and thawed immediately before use when its strength was adjusted with normal saline to lie between 80–120 mg. fibrinogen per 100 ml.

Collection of Plasma.—Blood was withdrawn by venepuncture into a clean dry syringe and added to 2% potassium oxalate or 3.8% sodium citrate in the proportion of one part of anticoagulant to nine parts of blood. It was then centrifuged at 1,000 revolutions per minute for 10 minutes to obtain plasma.

Collection of Serum.—From the same syringe four samples, each 1 ml., were added to four clean, dry standard test-tubes ($2\frac{1}{2}$ in. × $\frac{3}{5}$ in.) which were immediately incubated in a water bath at 37° C. The tubes were tilted every half minute until coagulation occurred, and the time between the first appearance of blood in the syringe and the coagulation was recorded as the Lee and White coagulation time. The tubes were then left undisturbed until one hour after coagulation had occurred, when the clot was gently separated from the side of the tube. The tubes were centrifuged at 1,000 revolutions per minute for three minutes and the serum separated.

Prothrombin Consumption Test Using Serum from Whole Clotted Blood

This test* is devised to measure the amount of prothrombin present in the original plasma as well as the amount remaining in the serum one hour after the blood has

*Modification of test devised by Rosemary Biggs.

clotted, the plasma and the serum being tested separately. In each case a two-stage technique is used. Any prothrombin which is present in the plasma or serum is converted into thrombin by the addition of an excess of brain thromboplastin and an optimum amount of calcium, and it is assumed that the amount of thrombin formed is proportional to the amount of prothrombin which was present originally. The amount of thrombin formed is measured by transferring measured amounts of the original incubation mixture to other tubes containing fibrinogen and the time taken for these latter tubes to clot is recorded. The more thrombin generated in the incubation mixture the more rapidly will the fibrinogen be clotted. By this means the amount of thrombin formed from the plasma and serum can be measured and the amount of prothrombin which each originally contained can be inferred.

Technique.—The standard test-tubes ($2\frac{1}{2}$ in. $\times \frac{3}{8}$ in.) were used throughout and the whole test was carried out at 37° C. To 0.2 ml. plasma (or serum) 0.2 ml. 0.85% sodium chloride and 0.2 ml. brain thromboplastin is added. As rapidly as possible thereafter 0.2 ml. $\frac{M}{40}$ calcium chloride is added, starting a stop-watch at this instant. The contents of the test-tube are mixed by inversion. Samples, each 0.1 ml., are removed from this mixture at 10 seconds, 20 seconds, 60 seconds, 120 seconds, and 180 seconds after the calcium has been added by means of a pipette calibrated to "blow out." Each of these samples is added to separate tubes containing 0.4 ml. fibrinogen, and the time which elapses between the addition of the 0.1 ml. of the incubation mixture to the fibrinogen and the coagulation of this mixture is noted. The end point is taken as that at which the first strands of fibrin appear.

In performing the test it is advantageous to have the assistance of one other person. The first observer prepares the incubation mixture and transfers the 0.1 ml. specimens to the fibrinogen solution. The second observer times the clotting of the second series of tubes, starting a second stop-watch at the instant the 10-second sample is added to the fibrinogen. With a little practice it is perfectly possible for one person to do the whole test.

When the plasma is tested it will be noted that a clot forms in the incubation mixture about 14 to 18 seconds after the calcium has been added. This is because the mixture is similar to the Quick one-stage prothrombin test. This clot needs to be expressed around a wooden applicator before the later specimens can be obtained. The 20-second specimen is usually removed from the incubation mixture just before the coagulation of the mixture. Owing to the absence of fibrinogen no such difficulty is encountered in the case of normal serum, but is occasionally met with in abnormal sera, especially haemophilic.

In a typical normal case the following results were obtained (Table I).

TABLE I

PROTHROMBIN CONSUMPTION TEST: TYPICAL RESULT IN A NORMAL INDIVIDUAL

Incubation time (secs.) ..	10	20	60	120	180
Plasma time (secs.) ..	30	18	16	25	46
Serum time (secs.) ..	220	165	160	205	242

The prothrombin consumption index would be recorded as:

$$\frac{\text{Most rapid plasma time}}{\text{Most rapid serum time}} \times 100$$

or in the example quoted:

$$\frac{16}{160} \times 100 = 10\%$$

The most rapid time is usually recorded in the 20- or the 60-second specimen, though occasionally one of the other specimens gives the shortest time. In most cases the time usually lengthened after the 60-second specimen, presumably from the action of antithrombin.

In normal cases the prothrombin consumption index has always been under 40%. In practically all cases of haemophilia and in blood rendered artificially thrombocytopenic by high-speed centrifugation, the prothrombin consumption index has been over 100% and in many cases even exceeding 150%. In these cases, therefore, the time taken for the incubation mixture derived from the serum to clot the fibrinogen has been less than that derived from the plasma. In other words the serum appeared to contain more prothrombin than the plasma. This phenomenon was noted by Bordet and Delange (1912) in the case of thrombocytopenic blood, and by Quick *et al.* (1949a) in both thrombocytopenia and haemophilia. It can also be inferred from the data published by Alexander and de Vries (1949b) in half of their cases. Quick *et al.* (1949a) commented that this demonstrated how inaccurate was the estimation of prothrombin in serum. It appears most unlikely that there is more prothrombin in serum than in plasma. A similar apparent increase in prothrombin can be observed if ordinary plasma is clotted with thrombin and the " serum " tested, once the thrombin added had been neutralized. The exact nature of this phenomenon remains at present unexplained.

Prothrombin Consumption Test using Serum from Recalcified Citrate or Oxalate Plasma

The test can also be done on recalcified citrate or oxalate plasma. In this modification of the test 1.0 ml. plasma in a standard test-tube is recalcified and the time taken for the tube to clot is recorded as the calcium time. In the experiments recorded in this paper the plasma was recalcified with 20 c.mm. of molar calcium chloride. Since this solution is hypertonic, care must be exercised in its use. Alternatively, a larger amount of $\frac{\text{molar}}{40}$ calcium chloride can be used. One hour after coagulation has occurred the clot is expressed around a wooden applicator, and 0.2 ml. of serum is removed and tested in a similar manner to that applied to serum from whole clotted blood.

This adaptation allows for experiments to be made with mixtures of various plasmas. It has some limitations which can modify the results. In particular, the speed and duration of centrifugation need to be very carefully standardized since these factors will alter the number and physical characters of the blood platelets. The following experiment demonstrates the effect of increasing speed and duration of centrifugation in a specimen of normal plasma (Table II).

TABLE II

EFFECT ON PROTHROMBIN CONSUMPTION OF CENTRIFUGATION OF A SPECIMEN OF PLASMA AT SEVERAL SPEEDS

Speed and Duration of Centrifugation (R.P.M.× Minutes)	Calcium Time (Mins.)	Plasma Time (Secs.)					Serum Time (from Recalcified Citrate Plasma) in Secs.					Prothrombin Consumption Index (%)
		10	20	60	120	180	10	20	60	120	180	
1,000×5 ..	3½	30	27	14	15	40	360+	360+	360+	360+	360+	under 5
1,500×5 ..	3½						250	195	195	200	245	7
2,000×10 ..	5						110	70	70	140	170	20
3,000×10 ..	5½						140	120	53	65	72	26

As the speed and duration of centrifugation increased, the plasma became clearer and the number of platelets diminished. When each of these plasmas was recalcified less prothrombin consumption occurred corresponding with the decrease in platelets. This effect was only noted once the plasma had been recalcified, the uncalcified citrated plasma always giving the same readings whatever its content of platelets. The use of recalcified plasma introduces a number of variables which are not very easy to control. For this reason the simpler method is preferable—i.e., using serum from whole clotted blood.

In the performance of these prothrombin consumption tests it needs to be emphasized that the test is susceptible to small alterations in technical detail. In particular, a bad venepuncture will cause an abnormally low reading. Dirty test-tubes, wet test-tubes, tubes of different size, and varying amounts of filling and tilting of the tubes during coagulation of the blood will all cause different readings. For all these reasons the technique adopted should be rigidly followed and too much emphasis should not be laid on small differences in results.

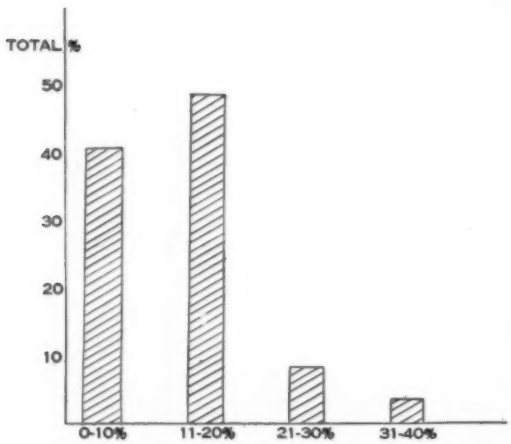

FIG. 1.—Prothrombin consumption index of 37 normal subjects.

Results

In Normals.—Observations on 37 normals are recorded in Fig. 1. These results were obtained by the use of serum from whole clotted blood. The subjects were mostly fit laboratory workers. The age and sex distribution of the controls are shown in Table III.

It will be noted that in the vast majority of cases the prothrombin consumption index is under 20% while all the

TABLE III

SEX AND AGE DISTRIBUTION OF NORMAL CONTROLS

Age in Years :	15–20	21–25	26–30	31–35	36–40	41–45	46–50
Males ..	2	2	5	4	1	—	1
Females ..	10	8	1	1	1	—	1
Total ..	12	10	6	5	2	—	2

normals fall in the range 0–40%. These figures compare well with those obtained by Alexander *et al.* (1949) where a one-stage technique was used, and the normal range was between 0–32% with the majority falling in the range 0–16%.

In Haemophilia and Thrombocytopenia.—Nineteen cases of haemophilia have been tested, and the results on the serum from whole clotted blood were as in Table IV.

TABLE IV

PROTHROMBIN CONSUMPTION IN 19 CASES OF HAEMOPHILIA

Case No. and Name	Age (Years)	Plasma Time (Secs.)					Serum Time (Secs.)					Prothrombin Consumption Index (%)
		10	20	60	120	180	10	20	60	120	180	
1 R.E.	9	16	20	17	37	75	50	40	40	105	180	40
2 A.B.	59	60	50	20	40	93	20	15	15	25	40	133
3 V.M.	12	33	38	27	46	105	17	15	18	30	45	180
4 W.D.	6	20	20	17	28	64	26	25	12	48	60	142
5 E.G.	50	25	94	36	60	100	20	20	25	40	70	125
6 P.R.	4	45	35	45	95	125	40	35	42	80	140	100
7 T.N.	38	25	20	20	45	45	60	50	25	15	32	133
8 J.D.	42	23	23	19	27	46	17	17	14	18	30	136
9 L.B.	11	20	19	23	55	61	15	20	23	44	85	127
10 J.O.	10	40	30	25	28	42	30	25	15	30	33	167
11 A.A.	50	23	25	22	37	58	18	25	30	50	95	122
12 T.E.	10	35	18	18	26	41	52	44	30	54	—	60
13 J.L.	50	40	23	21	29	43	37	22	22	32	60	95
14 A.H.	4	25	20	18	25	48	12	12	10	21	37	180
15 P.H.	18	30	23	20	28	42	20	17	25	38	66	118
16 P.L.	10	25	17	15	30	40	15	10	12	30	40	150
17 T.H.	10	25	22	16	26	49	15	10	11	15	25	160
18 M.J.	36	27	24	18	30	41	25	18	18	29	44	100
19 S.L.	50	26	20	15	35	42	30	20	23	27	35	75

It will be seen that in almost all cases values over 100% were obtained. In a few cases a little prothrombin consumption occurred. In the case R. E. it was noted that the venepuncture was not cleanly performed, but this did not apply in the other cases.

The test has been carried out in eight cases of naturally occurring thrombocytopenia. The results, using serum from whole clotted blood, are as set out in Table V.

TABLE V

PROTHROMBIN CONSUMPTION INDEX IN 8 CASES OF NATURALLY OCCURRING THROMBOCYTOPENIA

Case No. and Name	Disease	Platelet Count (per c.mm.)	Bleeding Time (Min.)	Purpura	Hess Test	Plasma Time (Secs.)					Serum Time (Secs.)					Prothrombin Consumption Index (%)
						10	20	60	120	180	10	20	60	120	180	
1 G.F.	Aplastic anaemia ..	4,000	20+	+	+	50	40	15	20	37	20	17	15	27	40	100
2 F.G.	Subacute myeloid leukaemia	Under 30,000	—	—	—	40	20	17	24	60	35	30	23	50	75	74
3 B.S.	Idiopathic thrombocytopenic purpura	0	25+	++	++	25	20	18	25	40	35	33	35	75	180	55
,,	14 days later	28,000	4	–	±	20	15	24	58	105	75	75	75	180	180	20
4 M.J.	Thrombocytopenia (due to gold)	6,000	15+	++	++	25	20	12	21	45	25	20	15	23	60	80
5 D.H.	Idiopathic thrombocytopenic purpura	11,000	15+	++	++	17	15	20	34	—	22	15	24	32	—	100
6 A.J.	Pernicious anaemia in relapse	80,000	4	—	—	30	22	16	31	57	290	240	220	300 +	—	7
7 D.P.	Thrombocytopenia purpura ? toxic	7,000	20	++	±	25	15	13	20	—	15	10	10	20	—	130
8 S.H.	Idiopathic thrombocytopenic purpura	12,000	30+	+	+	15	18	23	40	—	22	17	23	41	—	88

It will be seen that the prothrombin consumption defect is present in thrombocytopenia of varying aetiology. Provided the platelet count has been sufficiently reduced, the defect is present. Values over 100% are much rarer, possibly because some platelet fragments remain or because the platelets have not been entirely eliminated from the circulation.

If citrated plasma is centrifuged at high speed (15,000 r.p.m.) for 20 minutes, the supernatant plasma contains very few platelets. If this plasma is recalcified no prothrombin consumption occurs. In many cases values over 100% are obtained. In fact, if such a value is not obtained, the plasma should be recentrifuged, when almost invariably the result will exceed 100% (Table VI).

TABLE VI

PROTHROMBIN CONSUMPTION INDEX IN NORMAL PLASMA RENDERED THROMBOCYTOPENIC BY HIGH-SPEED CENTRIFUGATION

Source of Normal Plasma	Plasma Time (Secs.)					Serum Time (Secs.)					Prothrombin Consumption Index (%)
	10	20	60	120	180	10	20	60	120	180	
Donor A	50	30	17	16	35	15	15	20	30	55	107
,, B	22	14	22	37	60	14	13	17	28	—	108
,, C	22	14	22	37	60	14	13	17	28	—	108
,, D	26	16	16	19	38	14	11	12	19	50	145
,, E	30	16	14	19	35	15	15	21	34	—	93
,, F	17	14	15	19	24	18	14	16	18	38	100

It will be seen that in four out of six cases values over 100% were obtained, so that it appears likely that the mere removal of platelets has stopped prothrombin consumption. While the calcium time of these plasmas is usually prolonged to about eight to ten minutes they clot firmly so that an adequate amount of fibrinogen remains. In one case where the fibrinogen was estimated before and after high-speed centrifugation, it had fallen from 360 mg. per 100 ml. to 246 mg. per 100 ml. after 30 minutes at 17,000 r.p.m. The one-stage (Quick) prothrombin time, and the two-stage plasma time as measured in this test remained unaltered, so that it was unlikely that any material alteration in prothrombin or factor V had occurred in the plasma. It seemed fair therefore to ascribe the differences in behaviour of this plasma to the removal of the platelets.

TABLE VII

EFFECT OF MIXTURES OF NATURALLY OCCURRING THROMBOCYTOPENIC PLASMA AND HAEMOPHILIC PLASMA ON PROTHROMBIN CONSUMPTION

Volume of Haemophilic Plasma (ml.)	Volume of Thrombocytopenic Plasma (ml.)	Prothrombin Consumption Index (%)
1.0	—	180
—	1.0	80
0.9	0.1	8
0.1	0.9	35
0.5	0.5	9

TABLE VIII

EFFECT OF MIXTURES OF ARTIFICIALLY PREPARED THROMBOCYTOPENIC AND HAEMOPHILIC PLASMA ON PROTHROMBIN CONSUMPTION

Volume of Haemophilic Plasma (ml.)	Volume of Thrombocytopenic Plasma (ml.)	Prothrombin Consumption Index (%)
1.0	—	108
—	1.0	108
0.9	0.1	30
0.1	0.9	50
0.5	0.5	21

In Mixtures of Thrombocytopenic* and Haemophilic Plasmas.—Fresh citrated haemophilic plasma was mixed with fresh citrated plasma from a case of subacute myeloid leukaemia with a platelet count of under 5,000 per c.mm. The mixture was recalcified and tested as previously described (Table VII).

The next experiment (Table VIII) was similar to the last, except that normal citrate plasma freed from platelets was used as a source of thrombocytopenic plasma.

Similar results were obtained with both experiments. Neither the haemophilic nor the thrombocytopenic plasmas were capable of consuming prothrombin. Yet mixtures of these two plasmas had this capacity. On the whole it seemed as if the platelet-free plasma was able to correct the haemophilic plasma more easily than the haemophilic plasma was able to correct the platelet-free plasma. For this reason it was decided to measure these defects quantitatively.

Quantitative Experiments.—In the first experiment decreasing amounts of normal plasma were added to haemophilic plasma and the mixtures recalcified as before (Table IX).

TABLE IX

EFFECT OF THE ADDITION OF DECREASING AMOUNTS OF NORMAL TO HAEMOPHILIC PLASMA

Volume of Haemophilic Plasma (ml.)	Volume of Normal Plasma (ml.)	Final Concentration of Normal in Haemophilic Plasma (%)	Prothrombin Consumption Index (%)
1.0	—	—	133
0.9	0.1	10	10
0.9	0.01†	1	17
0.9	0.001†	0.1	66
0.9	0.0001†	0.01	92

† Made up to 0.1 ml. with normal saline.

It will be seen that 10% of normal plasma is quite adequate, and 1% of normal plasma is also capable of exerting an almost maximal effect. Even 0.1% of normal plasma has some effect. Thus normal plasma is capable of correcting haemophilic plasma as far as the prothrombin consumption defect is concerned when added in very small amount. This effect is quite independent of the platelets added and can

* In order to simplify reading only the prothrombin consumption index has been recorded.

TABLE X

EFFECT OF ADDITION OF INCREASING AMOUNTS OF PLATELET-CONTAINING PLASMA TO PLATELET-FREE PLASMAS

Volume of Platelet-free Plasma (ml.)	Volume of Platelet-containing Plasma (ml.)	Prothrombin Consumption Index (%)
1.0	—	100
—	1.0	8
0.95	0.05	40
0.90	0.10	28
0.85	0.15	19
0.80	0.2	17.5
0.75	0.25	15

be obtained with platelet-free plasma. The amount of plasma required was found to be considerably less in this patient than that reported by Alexander and de Vries (1949b). Unlike at least two of the cases reported by these authors this patient had not been receiving plasma transfusions.

In the next experiment platelet-containing plasma was added to platelet-free plasma prepared as previously described. The blood from which the platelet-containing plasma was derived had been centrifuged at 2,000 r.p.m. for 5 minutes (Table X).

This experiment was performed on a number of occasions since one tube was often "out of step." Nevertheless the result was always the same; as the volume of platelet-containing plasma increased, so did the prothrombin consumption. This result, however, depended to some extent on the number of platelets in the platelet-containing plasma, and in general the faster the plasma was spun the less its effect. This was only to be expected since centrifugation at faster speeds removed the platelets more efficiently. If haemophilic plasma was used as a source of platelets, these

TABLE XI

EFFECTS ON PROTHROMBIN CONSUMPTION OF MIXTURE OF PLATELET-FREE AND PLATELET-CONTAINING NORMAL AND HAEMOPHILIC PLASMAS

	Volume of Citrated Plasma Recalcified (ml.)				Prothrombin Consumption Index (%)
	Platelet-free		Platelet-containing		
	Normal Plasma	Haemophilic Plasma	Normal Plasma	Haemophilic Plasma	
A	1.0	—	—	—	127
B	—	1.0	—	—	160
C	—	—	1.0	—	15
D	—	—	—	1.0	127
E	0.1	—	—	0.9	20
F	—	—	0.1	0.9	17
G	0.5	0.5	—	—	120
H	—	0.9	0.1	—	75
I	—	0.8	0.2	—	60
J	—	0.7	0.3	—	22

platelets were as good as, if not better than, normal platelets. It will be seen that the volumes which require to be added to platelet-free plasma to correct the prothrombin consumption defect are very much larger than those required to correct the defect in haemophilic plasma. This would then be an additional argument in favour of the identity of the two factors which are the cause of the prothrombin consumption defect in these two types of plasma.

As a final check of the correctness of the hypothesis that at least two factors are required for the consumption of prothrombin, the experiments were repeated using both platelet-containing and platelet-free normal and haemophilic plasma (Table XI).

Rows A to D show that the only plasma capable of consuming prothrombin is the platelet-containing normal plasma. Rows E and F show that either platelet-containing or platelet-free normal plasma is capable of correcting a platelet-containing haemophilic plasma, while row G shows that even a much larger quantity of platelet-free normal plasma is incapable of correcting a platelet-free haemophilic plasma.

Rows H to J demonstrate once again that the platelets require to be added in sufficient amount to haemophilic plasma before adequate prothrombin consumption occurs.

It would thus appear that both platelets and the addition of a small amount of normal plasma are required in haemophilic plasma before adequate prothrombin consumption occurs, normal or haemophilic platelets being equally effective.

Discussion

The experiments recorded in this paper substantially confirm the work of the authors quoted earlier. Whatever else may be needed for adequate prothrombin consumption, a sufficient number of platelets is necessary as well as a small amount of a substance present in normal plasma. In both thrombocytopenia and haemophilia defective prothrombin consumption occurs. Thrombocytopenic normal plasma contains a vast excess of the factor which is capable of correcting haemophilic plasma, and this could be the substance which is present in normal plasma and known as "anti-haemophilic globulin." Only a very small amount of normal plasma, of the order of 1%, is required to be added to haemophilic plasma to render this indistinguishable from normal plasma in respect of its prothrombin consumption. On the other hand, platelet-containing haemophilic plasma can correct the defect in thrombocytopenic plasma provided enough platelets are added. In this respect haemophilic plasma behaves at least as well as normal plasma, which would be another argument in favour of the inherent normality of haemophilic platelets. The amount of platelet-containing normal plasma which needs to be added largely depends on the platelet content of the plasma, and thus varies from patient to patient and with the speed and duration with which the plasma has been centrifuged. This does not exclude the possibility that some platelets may be more efficient than others. Much more platelet-containing plasma is required to restore the prothrombin consumption of platelet-free plasma than is required to correct haemophilic plasma. These facts would tend to show that the defects in the two instances are different.

Summary

Observations have been presented on the prothrombin consumption defect present in thrombocytopenic and haemophilic plasma.

Whereas in normal subjects less than 40% of the prothrombin present in the plasma remains in the serum one hour after coagulation of the blood has occurred, in these two conditions anything over 70% may be present. In many of these cases the apparent amount of prothrombin in the serum is greater than in the plasma.

After removal of platelets from normal plasma by high-speed centrifugation, prothrombin consumption ceases.

By mixing the plasmas of haemophilia and thrombocytopenia it can be shown that each of these is capable of correcting the defect in the other. Furthermore, the quantities which need to be added in the two instances are not the same, and these two facts appear to be in favour of the separate identity of these two substances.

The addition to haemophilic plasma of quantities of normal plasma comparable to those which correct the defect in the coagulation time, of the order of 1%, prove equally adequate with regard to prothrombin consumption. It is likely that the substance acting is the same in both instances and is probably "antihaemophilic globulin."

In platelet-free plasma, platelets need to be added in very much larger quantity before the defect is corrected. Depending on the speed and duration of centrifugation, between 10% and 30% or more may be needed. In this respect haemophilic platelets are as good as normal platelets.

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REFERENCES

Alexander, B., and de Vries, A. (1949a). *Blood*, **4**, 747.
—— —— (1949b). Ibid., **4**, 752.
—— —— and Goldstein, R. (1949). Ibid., **4**, 739.
Bordet, J., and Delange, L. (1912). *Ann. Inst. Pasteur*, **26**, 737.
Brinkhous, K. M. (1939). *Amer. J. med. Sci.*, **198**, 509.
Jaques, L. B. (1943). *Biochem. J.*, **37**, 344.
Quick, A. J. (1947). *Amer. J. med. Sci.*, **214**, 272.
—— Shanberge, J. N., and Stefanini, M. (1949a). Ibid., **217**, 198.
—— —— —— (1949b). *J. Lab. clin. Med.*, **34**, 761.
Soulier, J. P. (1948). *Rev. Hémat.*, **3**, 302.

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URINARY EXCRETION OF CARBONIC ANHYDRASE

A SIMPLE TEST FOR THE DETECTION OF INTRAVASCULAR HAEMOLYSIS*

BY

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Roughton (1935) pointed out that in human blood, carbonic anhydrase is confined to the erythrocytes and none can be detected in the plasma. Indeed the absence of the enzyme from the plasma contributes to its effectiveness in promoting the transport of CO_2 as bicarbonate. As shown by Meldrum and Roughton (1933) carbonic anhydrase is also absent from normal urine, though it has since been detected in the renal parenchyma by Davenport and Wilhelmi (1941). Unpublished experiments of Roughton and Winton quoted by Roughton (1935) showed that when the dog's kidney was perfused with defibrinated blood, the serum of which contained free carbonic anhydrase and haemoglobin, both escaped into the urine, carbonic anhydrase more readily than haemoglobin. It therefore appears that the absence of carbonic anhydrase from normal urine is correlated with the intracellular location of the renal carbonic anhydrase, and its absence from the plasma.

Extravascular haemolysis is a physiological process. The absence of carbonic anhydrase from normal plasma therefore suggests that when erythrocytes are destroyed within the cells of the reticulo-endothelial system, their carbonic anhydrase is also destroyed, or is otherwise prevented from reaching the plasma. Intravascular haemolysis occurs much more rarely in association with certain forms of haemolytic anaemia. In these conditions it might be expected that carbonic anhydrase would be liberated into the plasma and excreted in the urine. Intravascular haemolysis may lead to haemoglobinuria, and may also be detected by finding methaemalbumin in the plasma. It does not seem to have been suggested that intravascular haemolysis might more easily be detected by finding carbonic anhydrase in the plasma or urine. This suggestion has been submitted to a preliminary trial by examining the urines of three patients attending Addenbrooke's Hospital, Cambridge.

Case Reports

Case 1.—A young man of 19 years was admitted under Sir Lionel Whitby with severe acute haemolytic anaemia and sensitized red blood cells. For several days he had haemoglobin in his urine, but it never contained erythrocytes. Thirteen specimens were examined over a period of eight days, during which a splenectomy was performed. This was followed by rapid clinical improvement.

* Preliminary communication.

Case 2.—A woman of 33 years was attending the out-patient department under Sir Lionel Whitby on account of nocturnal haemoglobinuria (Marchiafava-Micheli syndrome). Her haemoglobinuria was usually slight. A specimen of her urine during her last attack was examined.

Case 3.—A man of 37 years was admitted under Dr. L. B. Cole with a typical personal and family history of familial acholuric jaundice. His urine was examined during a haemolytic episode of moderate severity associated with well-marked icterus.

Method

The urines were examined by the simple semi-quantitative colorimetric method described by Brinkman (1933) and slightly modified by van Goor (1934). The only special apparatus required is a Y-shaped tube (Fig. 1). Each limb holds a little over 1 ml. and has a glass stopper at its upper end. The stem holds a little over 2 ml. and contains enough mercury to fill it and also to close the lower ends of the two upper limbs of the Y. The tube stands in a beaker, immersed up to the upper ends of the limbs in water and crushed ice.

Two solutions are required:—

(1) 0.02M $NaHCO_3$ coloured a pale red with phenol red, and boiled for 2 minutes to expel dissolved CO_2.

(2) 0.005M CO_2, prepared by placing 5 ml. 0.1M $NaHCO_3$ in a 100 ml. volumetric flask, adding about 80 ml. of distilled water followed by 5 ml. 0.1M HCl, and then diluting to 100 ml.

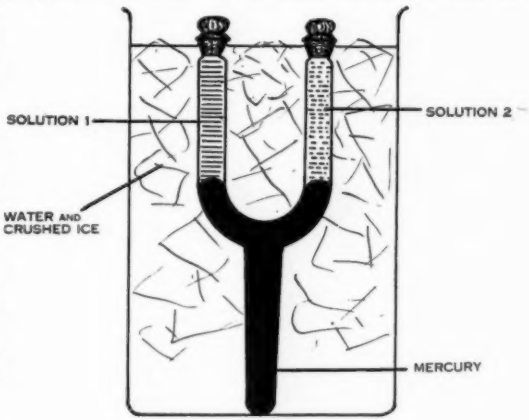

FIG. 1

Each limb of the Y-tube is filled with one of these solutions and the tube left in the ice for about five minutes for its contents to be chilled. The tube is then inverted a few times to mix the solutions, and replaced in the ice. A stop-watch started at the moment of the first inversion serves to measure the time taken for the CO_2 to be converted to carbonic acid and change the colour of the indicator from red to yellow. The disappearance of the last trace of pink gives a fairly sharp endpoint, the reaction being complete in about 120 seconds when the reagents are used alone.

Brinkman's practice was to add material to be tested for carbonic anhydrase to the CO_2 solution. Van Goor preferred to add it to the bicarbonate solution, and his method was used, with a modification to compensate for the acidity of the urine. Urine, 0.2 ml., was placed in a small tube with 0.8 ml. of the bicarbonate solution, and N/20 NaOH added from a fine Pasteur pipette until the original colour of the phenol red was restored. Further quantities of the bicarbonate solution were then added to give any desired dilution of the urine (1 ml. for 1 in 10; 4 ml. for 1 in 25; and 19 ml. for 1 in 100, for example). The test was then carried out exactly as before, except that the bicarbonate solution containing the diluted urine was used in place of the original solution. In most cases a dilution of 1 in 100 was found

suitable for urines that showed activity. Urines which showed no shortening of the reaction time at a dilution of 1 in 10 were considered not to contain carbonic anhydrase. When activity was found, a control test was made with a 1 in 10 dilution of the same urine after boiling for two minutes. Pasteur pipettes were used for introducing and removing solutions, and for washing with distilled water between tests.

Brinkman's criterion for the absence of carbonic anhydrase was that the reaction time should be at least as long as that found using the reagents alone. It was found in this work that urines classed as inactive, and previously active urines which had been boiled, gave times considerably longer than the control time. This is presumed to be due to slowing of the *p*H change by the urinary buffers. Because of this, and of the further possibility that some urinary constituents may be inhibitors of carbonic anhydrase, activities where found may have been underestimated, and the method cannot be claimed to be quantitative.

Results

Normal urines and that from Case 3 (acholuric jaundice) showed no activity. The time required for the colour change was always greater than 120 seconds even with the urine at a dilution of 1/10.

The urine from Case 2 (nocturnal haemoglobinuria), at a dilution of 1/100, reduced the reaction time to 40–44 seconds. A 1/10 dilution of this urine after boiling increased the time to 180 seconds approximately, the colour finally changing so slowly that the endpoint was hard to define.

Urines from Case 1 during his illness gave the following series of reaction times.

Date and Time of Collection	Dilution	Reaction Time (Secs.)
Oct. 16, 1949 .. 5.00 a.m.	1/100	14
,, 16 .. 10.30 a.m.	1/100	10
,, 16 .. 5.00 p.m.	1/100	8
,, 16 .. 9.00 p.m.	1/100	16
,, 17 .. 6.30 a.m.	1/100	21
,, 17 .. 5.30 p.m.	1/100	36
,, 17 .. 9.45 p.m.	1/100	36
,, 17 .. 11.15 p.m.	1/100	50
,, 18 .. 2.00 a.m.	1/100	40
,, 18 .. 6.30 a.m.	1/100	80
,, 18 .. 6.30 a.m.	1/25	40
,, 19 .. Splenectomy		
,, 20	1/10	Greater than 120
,, 22	1/10	,, ,, 120
,, 24	1/10	,, ,, 120
Control time with reagents alone:	120 seconds	

In no case were erythrocytes found in centrifuged deposits. Dilutions (1 in 10) of the active urines after boiling all gave times greater than 120 seconds. The last three urine specimens before splenectomy did not show haemoglobinuria. The most active urine in a dilution of 1 in 400 gave a reaction time of 30–34 seconds. A similar time was reported by van Goor for a 1 in 4,000 dilution of blood. Hence this urine appears to have contained at least one-tenth as much carbonic anhydrase as its own volume of laked blood. It is interesting to note that no carbonic anhydrase was found in the urine of this patient after splenectomy.

These observations suggest that the examination of the urine for carbonic anhydrase may provide a useful test for the differentiation of the haemolytic anaemias with intravascular haemolysis from those in which haemolysis is extravascular. The test is very simple, takes only a few minutes, and requires only a few millilitres of urine. Provided that care is taken to exclude menstrual contamination or haematuria with subsequent lysis of erythrocytes in a dilute urine, it would be expected to be specific.

Summary

A simple test for intravascular haemolysis is described, based upon the detection of carbonic anhydrase in the urine.

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REFERENCES

Brinkman, R. (1933). *J. Physiol.*, **80**, 171.
Davenport, H. W., and Wilhelmi, A. E. (1941). *Proc. Soc. exp. Biol., N.Y.*, **48**, 53.
Goor, H. van (1934). *Bijdragen tot kennis van de Koolzuuranhydrase.* Utrecht.
Meldrum, N. U., and Roughton, F. J. W. (1933). *J. Physiol.*, **80**, 113, 143.
Roughton, F. J. W. (1935). *Physiol. Rev.*, **15**, 251.

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FORTUITOUS FACTORS AFFECTING THE LEUCOCYTE COUNT IN BLOOD FROM THE EAR

BY

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The technique of a leucocyte count comprises not only accurate counting of cells but also taking a reliable sample of blood so that the cell picture reflects that found in the general circulation. It is usual for textbooks to enumerate the sites for skin puncture, and they may mention one or more of the following points: (1) wiping away the first drop of blood, (2) use of alcohol or ether for cleaning the skin, (3) rubbing to cause hyperaemia, and (4) avoidance of squeezing.

Choice of Blood Drop

It is frequently advised that the first drop or two of blood should be wiped away and a subsequent one used, but the reason for doing this, namely, skin contamination of the first few drops, is only mentioned by Gulland and Goodall (1925). In practice no special care need be taken against skin contamination. Theoretically a few squamous cells or extraneous matter might conceivably contaminate the drop, but do not affect the blood count.

Under certain circumstances, however, a very real reason exists for not using the first drop of blood from the ear which, if not realized, may lead to serious errors in the leucocyte count. Lucey (1921) pointed out the importance of seemingly unimportant differences in technique on the percentage monocyte count. Two people taking a blood film from an ear of the same man, both films being counted by one individual, could produce very different results in the number of monocytes present, variations from 5 to 40% being encountered. The first drop from the ear usually showed a high monocyte count, whereas subsequent drops gave normal counts. These films were made by pricking the skin without subjecting it to any preliminary treatment such as cleaning with alcohol or ether, or rubbing or massaging it to cause hyperaemia.

Site of Puncture

The two sites commonly used in adults are the ear and finger. Many haematologists prefer the ear, the advantages claimed for it being that it yields successive drops of blood more readily, is out of sight of the patient, and is less sensitive.

Table I shows a series of observations on healthy men and malarial patients comparing the monocyte counts in the first and third drops from a single puncture from each of the four places usually chosen for obtaining blood drops: the lobule of

TABLE I

ANALYSIS OF MONOCYTE COUNT IN NORMALS AND MALARIA PATIENTS

Case	Percentage of Monocytes								
	Lobule of Right Ear		Border of Right Ear		Base of Nail		Pulp of Finger		Vein
	Drop 1	Drop 3	Drop 1	Drop 3	Drop 1	Drop 3	Drop 1	Drop 3	
1. Normal	**24**	5	**20**	6.5	7	7	5.2	6	5.5
2. Normal	**35**	7	**41**	8.5	6.5	8	6	5.5	7
3. Normal	8	8	**27**	6.5	7	7.2	5.5	8.2	6.5
4. Normal	**22**	11	10	10	9.2	12	11	9.5	9.5
5. Normal	9.5	7	6.5	7.5	6	6	8	5.2	6
6. Normal	10	9.2	**16**	8.5	12	10	9.5	11	8
7. Malaria. *Plasmodium vivax*. Heavy infection. Late amoeboid forms and schizonts ..	**28**	**20**	**37**	**21**	**20**	**20**	**22**	**21**	**19**
8. Malaria. *Plasmodium vivax*. Scanty infection. Very few early amoeboid forms ..	**32**	9	**31**	8.5	9.5	8	7.5	8.5	8.5
9. History of malaria. No parasites found. Bout of fever ending 3 days before taking blood films	**19**	**17**	**33**	**15**	**17**	**19**	**16**	**17**	—

ear, border of ear, base of finger-nail, and pulp of finger. Blood was also taken from a vein. The first drops from the ear were kept small, and care was taken not to stimulate the local circulation before the prick was made. The ear was supported on the finger rather than grasped firmly, and rubbing the skin with alcohol or ether or massaging the part were avoided. Four hundred cells were counted in each film.

Results

Our analysis shows that in blood taken from the ear of normals the monocyte count may vary in the first drop taken from different parts, lobule or border, of the same ear. It may be normal in one and raised in the other (Cases 3, 4, and 6). There may be no monocyte increase in the first drop from either lobule or border (Case 5). If the monocyte count is raised, this is found in the first drop but not in the third.

Whether the blood is taken from the base of the nail or pulp of the finger the monocyte count is not raised in either the first or third drops. The counts correspond not only with each other but with counts from the third drops taken from the ear.

The count in venous blood corresponds with any drops from the finger and with the third drops from the ear.

True and False Blood Pictures.—These results indicate that monocyte counts from the vein, the finger, and the third drops from the ear represent true pictures,

whereas those from the first drop from the ear, when raised above normal, are false pictures.

Two cases with malaria (Cases 7 and 9) showed a true monocyte increase from all sites and an added higher count from the first drops from the ear, and one patient (Case 8) showed a normal count except from the first drops from the ear.

Blood was taken from the big toe, heel, leg, forearm, and tip of the nose. In none of these sites was a difference found in the monocyte count in the first and third drops.

Explanation of the Phenomenon

The ear in man is exposed to changes of temperature and, being an immobile appendage, its circulation is not aided by muscular contraction. In this second respect it is, as an external organ, unique ; for long periods it may not be subjected to warmth, pressure, or any speeding-up of its circulation following an increase in the rate of the general circulation through exercise. In these circumstances its capillary circulation slows down, and the monocytes then tend to cling to and become concentrated along the capillary walls. This concentration, when tapped by puncture, appears in the first drop of blood, and exceptionally, if the first drop taken is a small one, is also present in the second drop but not in the third drop, which represents a refill from the arterioles and is a representative sample of blood in the general circulation. Experiments have shown that when the circulation in the ear is rendered brisk by rubbing, massaging, or flicking it, or by applying heat in the form of a rubber hot-water bottle, the cell concentration is washed into the general circulation and the first drop then becomes a reliable sample of blood. The mere act of grasping the lobule firmly, and expressing a drop or two of blood, may sufficiently stimulate the circulation at this spot so that when a second puncture is made in the immediate locality the monocyte count in the first drop from the second puncture is low compared with the count in the first drop from the first puncture. Exercise, such as bending down several times to touch the toes, was found to disperse the cells.

Effect of External Temperature and Body Posture.—All these observations were made when the air temperature was moderate or cool, and on men who were up and about. When, however, differential counts were made on blood from the ears of outdoor workers during a spell of hot summer weather no concentration of monocytes in the first drop was observed, nor was it found in the winter months among men working in warm rooms.

Patients who are in bed do not show this concentration as the recumbent position, warmth, and occasional pressure of the pillow on the ears all tend to keep the local circulation brisk. It was, however, observed in one bed patient who had been sitting up for several hours near an open window.

Total and Absolute Leucocyte Counts

Numerous counts were made to determine to what extent when the ear circulation was sluggish, as indicated by a monocyte concentration in the first drop, the differential percentage, total leucocyte, and differential absolute counts were affected.

To bring out clearly any excess of leucocytes in the first over the third drop it was desirable that this first drop should be kept as small as possible because a monocyte concentration tails off as the drop enlarges. Punctures were therefore made which yielded a drop of blood just sufficient to fill a " Thoma " white-cell pipette

TABLE II

	Case	No. of Drop	Differential Percentage Counts				Total Leucocytes Normal (5,000–10,000 per c.mm.)	Differential Absolute Counts			
			Monocytes Normal (2–12%)	Neutrophils Normal (50–70%)	Lymphocytes Normal (20–35%)	Eosinophils Normal (1–4%)		Monocytes Normal (200–1,000 per c.mm.)	Neutrophils Normal (3,000–6,500 per c.mm.)	Lymphocytes Normal (1,250–3,250 per c.mm.)	Eosinophils Normal (50–400 per c.mm.)
Ear not Rubbed or Massaged before Puncture	1	First	**29.2**	37.5	32.2	1.0	8,875	2,591	3,328	2,857	88
		Third	9.0	57.0	32.2	1.7	5,000	450	2,850	1,610	85
	2	First	**25.2**	41.2	32.5	1.0	13,250	3,339	5,459	4,306	132
		Third	6.7	57.2	35.0	1.0	7,125	477	4,075	2,493	71
	3	First	**30.0**	45.0	23.0	2.0	21,000	6,300	9,450	4,830	420
		Third	7.0	65.0	27.0	1.0	10,000	700	6,500	2,700	100
Massaged before Puncture	4	First	6.7	59.2	32.5	1.5	8,000	536	4,736	2,600	120
		Third	7.0	62.0	29.5	1.5	8,250	577	5,115	2,433	123

to the 0.7 mark. The blood was then expelled on to the end of a microscope slide so as to mix the cells thoroughly, and part was drawn up again into the pipette to the 0.5 mark and diluted 1:20 with 1% acetic acid solution. The remainder left on the slide was spread as a film. In this way the same mixture of cells for both total and differential counts was assured. The spreader used was narrower than the slide so that the horizontal edges of the film where the monocytes and polymorphs tend to accumulate could be included in the differential count. A second drop was then expressed and wiped away, and the third drop taken into another "Thoma" white-cell pipette and the same procedure repeated. Four representative counts are shown in Table II. They were taken from four healthy men. The site of puncture was the ear, body posture upright, and the external temperature cool. In the films 400 cells were counted in parallel vertical fields.

Results

The total count in the first drop is raised, but whether or not the rise constitutes a false leucocytosis depends not only on the degree of concentration of the cells in the capillaries of the ear but upon the level of the true count as shown in the third drop. Thus in Case 1, where the true count, 5,000 per c.mm., is a low normal count, the false count, 8,875 per c.mm., does not amount to a marked leucocytosis. But in Cases 2 and 3, where the true counts are 7,125 and 10,000 per c.mm., there is in the first drop a false leucocytosis of 13,250 and 21,000 per c.mm. respectively.

The absolute counts of all the leucocytes are raised, but the degree of increase is much the greatest in the case of the monocytes. These cells in Case 1 are increased nearly six times, in Case 2 seven times, and in Case 3 nine times, whereas the neutrophil polymorphs and the lymphocytes are in no case doubled.

In Case 4 a brisk circulation was assured before puncture by massaging the ear for three seconds. The counts are seen to be approximately the same in first and third drops.

Red Cell Count and Haemoglobin Content.—Numerous estimations showed that these were not affected even when there was a concentration of leucocytes in the first drop of blood from the ear. The sluggish stream allows the leucocytes to collect along the capillary walls, but the red cells evidently flow slowly on without becoming packed.

Effect of Alcohol or Ether on the Skin and Rubbing on Blood Count

Most haematologists advise the use of alcohol or ether, but others consider them to be harmful. If their purpose is to sterilize the skin they may as well be discarded, because all that is necessary to prevent sepsis is to use a sterile pricker. If their purpose is to remove the natural grease from the skin, again they should be discarded, because this grease facilitates the formation of a well-rounded blood drop which is an advantage.

According to Price-Jones (1933), "The auricle should be gently massaged and not rubbed: cleaning with iodine, ether, etc., is not necessary and should be avoided in view of the artificial hyperaemia, the possible leucocytic attraction, and introduction of tissue juice likely to be set up thereby."

Gulland and Goodall (1925) conclude that "elaborate antiseptic precautions are unnecessary, and cleaning with ether should be avoided."

I have tested both alcohol and ether in this respect and do not find that they affect the blood count in any way, but I consider them unnecessary and do not use them.

Hyperaemia is desirable as it promotes a free flow of blood from the puncture. It is most conveniently produced by massaging the part between finger and thumb for three seconds. In the case of the ear this procedure has the additional advantage of dispersing any concentration of leucocytes which may be present. To make assurance doubly sure the first drop or two of blood should be wiped away.

Summary

The ear in man, owing to atrophy of its muscles, is immobile, hence its capillary circulation is not aided by muscular contraction, and in the absence of external warmth, pressure, bending movements, or a speeding-up of its rate of flow secondary to exercise, its capillary circulation tends to slow down. When this happens the leucocytes start concentrating along the capillary walls.

The various kinds of leucocytes are not concentrated to the same extent: the monocytes cling to the capillary walls far more than other cells. Samples of blood under such conditions show distorted differential percentage and absolute counts, and a raised total leucocyte count which may amount to a false leucocytosis.

The concentration of leucocytes when tapped by puncture is found in the first drop of blood from the ear. It is not found in the third drop, which represents a refill from the arterioles and gives a true picture as found in the general circulation of the leucocytes.

A high percentage of monocytes in the first but not in the third drop is a sure indication that the first count is due to a fortuitous concentration. Such a concentration can be dispersed by massage, warmth, bending down, or exercise.

External temperature affects the capillary circulation of the ear. A leucocyte concentration occurs in moderately cool and cold weather but not in hot weather, nor is it found in persons working in warm rooms. Body posture affects it. It is not found in recumbent bed-patients, but it is likely to occur among out-patients.

To avoid this error the part of the ear to be pricked should be massaged for three seconds and, as an extra precaution, the third drop taken.

In the case of the finger the capillary circulation is kept brisk by muscular contraction and movement of the part, consequently the leucocyte picture is reliable whichever drop is examined.

REFERENCES

Gulland, G. L., and Goodall, A. (1925). *The Blood.* Green. Edinburgh. P. 1.
Lucey, H. C. (1921). *Trop. Sect. Proc. R. Soc. Med.*, **14**, 53.
Price-Jones, C. (1933). *Blood Pictures.* Wright. Bristol. P. 8.

[The author does not take into account the possibility of local endothelial activity, which certainly plays some part in the production of this effect, especially in acrocyanosis associated with bacterial endocarditis.—ED.]

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TECHNICAL METHODS

THE IDENTIFICATION OF BARBITURATE DRUGS IN GASTRIC CONTENTS AND URINE

BY

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Cases are admitted from time to time to most large hospitals in a state of coma or semi-coma in which the differential diagnosis includes overdosage with the barbiturate series of narcotic drugs. It is often important to confirm or exclude this factor as quickly as possible. Standard methods of analysis (e.g., the Stas-Otto process, Smith, 1934a), although ultimately giving accurate quantitative results, are time-consuming.

An endeavour has been made to evolve a more rapid method of analysis which is sufficiently delicate to give an answer to the question of whether or not barbiturate poisoning plays a significant part in the clinical condition of the patient. The method in our hands takes about three hours to perform and is based upon the facts that (1) the barbiturate drugs, being derived from barbituric acid, are either the acid derivatives, e.g., diethylbarbituric acid (barbitone), or their sodium salts, e.g., sodium diethylbarbiturate (soluble barbitone). (2) When in aqueous solution they can be changed from one form to the other by making the *p*H acid or alkaline. (3) The barbituric acid derivatives are only slightly soluble in water but are readily soluble in ether, whereas their sodium salts are readily soluble in water and almost insoluble in ether.

As part of any ingested barbituric acid is excreted unchanged in the urine, at least 100 ml. of urine are collected and examined in all cases. Unless the state of coma is known to have lasted for a long time, the gastric contents are aspirated and examined as well.

Method of Extraction

The sample is tested with litmus paper and if necessary made acid with concentrated hydrochloric acid drop by drop and then evaporated to small bulk on a boiling-water bath ; e.g., 100 ml. is reduced to 15 ml. Frequent stirring may be necessary to prevent charring of the mucoid deposit on the side of the evaporating dish. The residue is transferred quantitatively with two washings of 2 ml. distilled water to two large centrifuge tubes (20–50 ml. capacity), half in each, and extracted three times with equal volumes of ether. Centrifuge tubes are used, as any emulsion formation can be quickly broken down by spinning and the supernatant ether removed with a Pasteur pipette. The ether extracts, containing the barbituric acid if present, are filtered and evaporated

to dryness. The total residue is stirred with 5–10 ml. *N*-sodium hydroxide to form a solution of barbiturate, the insoluble impurities filtered off, and the filtrate just acidified to litmus paper with concentrated hydrochloric acid. If much fat were present in the ether residue, the alkaline solution is put in the refrigerator, and when cold most of the fat adheres to the sides. The solution is then filtered again when the remainder of the fat adheres to the filter paper. If much barbituric acid is present, a white precipitate appears on acidifying, but faint precipitates often occur with normal extracts. This mixture is extracted with ether as before, the extracts being filtered into a tube containing about 1–2 g. anhydrous sodium sulphate. After mixing well and allowing the sulphate to settle, the dried ether is filtered, together with ether washings of the sulphate, into a tube containing about 0.5 g. activated charcoal. After mixing well and allowing the charcoal to settle, the ether is filtered through a funnel containing a layer of charcoal about 1 cm. deep supported on a cotton-wool wad. The ether filtrate, freed of any pigments, and ether washings of the charcoal are evaporated to dryness. A barbituric acid may appear as white crystals or as a clear, colourless gum which slowly crystallizes into needles or rosettes.

Tests on Ether Residue

Cobalt Test.—A few crystals or a drop of the gum are dissolved in a few drops of absolute ethyl alcohol, 6 drops of 5% *iso*propylamine in absolute methyl alcohol are added, and then one or two drops of 0.5% cobaltous acetate in absolute methyl alcohol. In the presence of a barbituric acid, a violet colour is obtained, otherwise only a barely discernible pink colour is given. (Thiobarbituric acid derivatives such as pentothal give a paler and pinker colour, the sodium salt giving a green colour.) When only a small amount of residue is left after evaporating the final ether extract, the cobalt test is carried out directly on the residue as a whole. In this way 15 mg. (approximately grains ¼) phenobarbitone added experimentally to 100 ml. gastric juice were detected by a weak positive cobalt reaction, a violet colour fading within 30 seconds. Larger amounts gave a deep permanent violet colour.

If a good yield of cobalt-reacting crystals or gum is obtained from gastric contents a further purification as described below can be carried out, and the barbituric acid present in the sample estimated roughly as $3/2 \times$ weight of the final yield.

Similar extraction rates were obtained with urine samples, but the amount of the barbituric acid excreted in the urine and the rate of the excretion varies considerably with the different compounds, and presumably with the patient's kidney function as well. We have found that the ether residues (without the further purification) could roughly be arranged into three groups as a guide to the quantity of the drug consumed.

(1) Urine collected for about nine hours after a single dose of a barbiturate drug of 1½–3 grains (approximately 100–200 mg.) gave either a negative result or occasionally a weak positive as above. The cobalt test was performed on the whole of the ether residue, which was usually about 10 mg. in weight and obviously impure. Occasionally larger crystalline residues were obtained, but portions gave negative results and so the test was repeated on the whole.

(2) Doses of barbiturate of 5–10 grains (approximately 300–600 mg.) resulted in residues, sometimes showing characteristic crystal formation, which gave a deep permanent violet colour with the cobalt test.

(3) Doses of 15 grains (approximately 1,000 mg.) and upwards gave yields of 50–100 mg. or more, usually showing the characteristic crystal formation of the particular compound after evaporation of the ether (Turfitt, 1948). Portions of such residues gave positive cobalt reactions. We have so far obtained results such as these on all the four patients admitted during the past eight months in coma, later shown to have taken large quantities of a barbiturate drug. In these cases the urine was collected from the time of admission. In one case this was 36 hours after the onset of coma, and 500 ml. urine, collected by an indwelling catheter during 12 hours with intravenous fluid therapy, yielded 80 mg. barbitone.

Selenious Acid Test.—It was found that the selenious acid reaction (Turfitt, 1948) gave positive results with the ether available in this laboratory, similarly the salicyl aldehyde reaction (Smith, 1934b) gave positive results with absolute alcohol: consequently this test could not be used.

Identification of the Extracted Barbituric Acid

When sufficiently large residues are obtained further identification of the barbituric acid derivative is carried out as follows.

(1) The residue is further purified by extraction with N ammonium hydroxide, filtering, acidifying the filtrate, extracting with ether and continuing as before with sodium sulphate and charcoal. With amounts of 100 mg. (approximately gr. 1½) or more of a barbituric acid in 100 ml. gastric juice, the yield of crystals after this purification was 60–70%.

(2) The melting point of resulting crystals is determined. This enabled the substance to be tentatively assigned to one of four groups shown in Table I.

TABLE I

Barbituric Acid	Melting-point of Pure Substance (° C.)	Melting-point of Final Ether Residue (° C.)
Barbitone (" veronal ")	189–192	180–182
Phemitone (" prominal ")	178–181	169
Phenobarbitone (" luminal ")	173–177	170
" Phanodorm "	173	169–170
Allobarbitone (" dial ")	171–172	170–171
Thiopentone (" pentothal ")	156–159	156
" Amytal "	153–155	151
Hexobarbitone (" evipan ")	145–147	135
Pentobarbitone (" nembutal " is sodium salt)	127–130	125
Butobarbitone (" soneryl ")	127	118–120

(3) Further evidence is obtained from the three colour reactions (i) vanillin reaction (Turfitt, 1948); (ii) *p*-dimethylaminobenzaldehyde reaction (Turfitt, 1948); (iii) formalin reaction (Smith, 1934b).

It was found necessary to set up a full series of control tests with pure samples of the barbituric acids whenever these reactions were tried, as the shades of the colours seemed to vary with the rate and duration of heating. The formalin reaction

TABLE II

Barbituric Acid	Formalin Colour Reaction
Barbitone	Colourless
Phemitone	Red-brown
Phenobarbitone	Pink
" Phanodorm "	Orange
Allobarbitone	Yellow
Thiopentone	Pale yellow
" Amytal "	Deep yellow
Hexobarbitone	Deep yellow
Pentobarbitone	Yellow
Butobarbitone	Very pale yellow
Reagent control	Colourless

was originally described to distinguish phenobarbitone from barbitone and phemitone, but was found useful as a general colour reaction as shown in Table II.

For the test about 1 mg. of crystals are heated with 0.5 ml. formalin and 4 ml. concentrated sulphuric acid in a boiling-water bath for one minute. The crystalline forms of the barbituric acids are also described by Turfitt, and these provide additional evidence of identity.

(4) Final evidence of identity can be obtained by preparing a pure crystalline sample by crystallization from absolute alcohol or micro-sublimation. The melting point and mixed melting point (with known pure crystals) are then determined.

The barbituric acids extracted can usually be identified by the preliminary melting points and the colour reactions before proceeding to this final test.

Medicinal Tablets or Capsules

A tablet ground up or the powder from a capsule is stirred with a few millilitres of dilute hydrochloric acid and the mixture, after checking it to be acid to litmus, is extracted with an equal volume of ether. A portion of the filtered extract is tested by the cobalt test for the presence of a barbituric compound.

If a barbiturate is present, a pure sample is obtained by stirring the powder from a few tablets or capsules with a few millilitres of dilute hydrochloric acid, making certain that the mixture is acid, and extracting with ether. The extract is filtered, evaporated to dryness, and the residue treated as described above.

Specificity of the Cobalt Test

Parri (1924) described this reaction using cobalt and ammonia solution. We have used Koppanyi's method with *iso*-propylamine as it gives a more distinctive violet colour which does not disappear with excess *iso*-propylamine; excess ammonia removes the violet colour. Lapiere (1947) found that certain sulphonamides react with Parri's test. We found that sulphathiazole gave the characteristic violet colour, but not so deeply as the barbiturates, whilst sulphanilamide, sulphapyridine, and sulphadiazine gave a pale pinkish-violet colour. But sulphonamides

are soluble in acid solutions, and insoluble or only slightly soluble in ether, so that the extraction methods described above do not remove sulphonamides from the test material in sufficient quantity to give positive cobalt reactions. This has been checked by extracting 100 ml. gastric juice containing 5 g. sulphathiazole, and medicinal tablets containing 0.5-g. amounts of these four sulphonamides with negative results. Urine containing both free and acetylated sulphonamide also gave negative results ; the acetylated compound is hydrolysed to the free compound by the preliminary heating in acid solution.

Summary

A method of detecting barbituric acid derivatives in gastric contents, urine, and medicinal preparations is described. The method has been kept as simple and as rapid as possible while retaining sufficient sensitivity to enable it to be used in confirming the diagnosis in cases of coma with suspected barbiturate poisoning.

A scheme is presented for obtaining rough estimates of the amounts of barbiturate drug consumed. Therapeutic doses (up to 3 gr. or 200 mg.) by mouth gave negative or very weak positive results on urine analysis ; doses of 5–10 gr. (approximately 300–600 mg.) gave consistent positive results ; higher dosage gave good yields allowing identification of the particular compound. The method detects gr. $\frac{1}{4}$ or 15 mg. barbiturate in 100 ml. of urine or gastric contents, and with the presence of more than $1\frac{1}{2}$ gr. or 100 mg. per 100 ml. the yield after further purification was 60–70%.

The identification of ten barbituric acid compounds in common use is described.

We express our gratitude to Dr. L. H. D. Thornton for his advice and to Dr. G. E. Turfitt for valuable information.

References

Lapiere (1947). *An. chim. Acta*, **1**, 390.
Parri, W. (1924). *Boll. chim. farm.*, **63**, 401.
Smith, S. (1934a). *Taylor's Principles and Practice of Medical Jurisprudence*. 9th Ed., **2**, 335.
—— (1934b). Ibid., 9th Ed., **2**, 666.
Turfitt, G. E. (1948). *Quart. J. Pharm.*, **21**, 1.

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MULTIPLE MARROW CULTURES FROM A SINGLE ASPIRATE

BY

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Osgood and Brownlee (1937) described a simple method of bone marrow culture. They employed 20 ml. serum bottles containing a plasma or serum medium diluted with a physiological salt solution. Since the volume of marrow aspirate is limited to 1–2 ml., and it is best to have 1–2,000 cells per ml. of medium, one experiment is usually limited to five or six cultures. This method permits of as many as 30 cultures being explanted from one specimen of marrow.

Method

The media are prepared in serum bottles as in Osgood's method, but the cultures are carried out in 100 × 3 mm. tubes sealed at one end. This capillary bore prevents escape of the medium on inversion of the tubes. Using Pasteur pipettes the tubes are filled with medium, allowing 2.0 to 2.5 cm. for the marrow. The filled tubes are inverted in open sterile serum bottles and placed in readiness in an incubator at 37.5° C. If several tubes are filled with the same medium specimens may be examined at intervals.

Using a Salah needle, 1.5 to 2.0 ml. marrow is aspirated (larger samples tend to be unduly diluted with blood). The specimen is injected into a serum bottle containing Geys' solution at 37.5° C. and cellular masses are broken up by forcefully sucking the suspension to and fro from container to syringe. The marrow cells are then washed free of plasma by centrifuging and resuspending in fresh uncitrated Geys' solution. (The volume of Geys' solution used depends on the cellularity of the specimen and the number of tubes to be used in the culture.) Nucleated cell counts may be made at any stage. The final marrow suspension is then injected into the culture tubes, using a fine-bore syringe of the type used in tuberculin testing. The marrow cells are readily distributed throughout the tubes by inversion; a horizontal position during culture ensures an even distribution of cells.

After culture the tubes are centrifuged at 1,500 revolutions for 15 minutes, the medium is withdrawn, and smears are made from the cellular deposit.

In each of 10 such experiments satisfactory smears were obtained. Scrupulous care is required at every stage to prevent infection. The technique has been used for studying the conditions influencing the maturation of megaloblastic marrows.

Summary

A method is described whereby numerous cultures can be carried out on single specimens of aspirated bone marrow.

The author is indebted to Dr. Laslo Lajtha for instruction in the Osgood technique, and to Dr. C. C. Ungley for much help and for facilities in carrying out the cultures.

REFERENCES

Osgood, E. E., and Brownlee, I. E. (1937). *J. Amer. med. Ass.*, **108**, 1793.

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AN EASY TECHNIQUE FOR Rh GROUPING

BY

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The method of grouping described here is now in routine use in the serology laboratory, Royal Army Medical College. It requires only 0.0065 ml. (1/150 ml.) of serum for each test and the technique of setting up and reading is quick and easy. In addition the labour of washing up afterwards is almost eliminated.

Equal volumes of grouping serum and saline red cell suspension are mixed on a " perspex " surface which is then inverted and incubated in a moist atmosphere. The drop does not wet " perspex " and therefore hangs from the underside as a hemisphere. The red cells sediment in the drop and collect in the tip just as they do in a tube. After incubation a coverslip is applied and the preparation is examined microscopically for agglutination.

Apparatus

" Perspex " Slides.—These are made from odd lengths of " perspex " ; a convenient size is 10 cm. × 2.5 cm. × 0.5 cm. The front surface of each slide is divided into four equal areas by means of three transverse cuts made with a triangular file and extending part way through the thickness of the slide. Each area, about 2.5 cm. × 2.5 cm., is sufficient for one test, and the areas are conveniently numbered by engraving the back surfaces of the slides with a sharp instrument.

Moist Chamber.—This may be improvised from a petri dish. We use a box made from a " perspex " museum jar and provided with a close-fitting lid. Two strips are cemented along the inside long walls. The slides fit transversely across the box so that their extreme ends rest on the strips. A pad of moist blotting-paper is placed on the bottom.

Setting-up

Volumes are measured by calibrated capillary pipettes or finely drawn waxed droppers. In either case equal amounts (1/150 ml. or less) of grouping serum and 2% red cell suspension are taken up and deposited gently together in the middle of one of the areas. The mixed drop is stirred carefully with a glass needle without spreading the drop over the surface. When all tests have been set up, the slides are inverted and placed in the moist chamber so that the drops hang suspended from the under surface. The chamber is incubated at 37° for two hours.

Reading

Naked eye readings of the sedimented red cells are possible, but we always confirm such readings microscopically. The slides are removed from the chamber and held in the inverted position. Each drop is then lowered so that it contacts the centre of a ¼-in.-round coverslip, which is drawn up by capillary attraction into contact with the

slide. The slide is then turned right side up and examined under the low power of the microscope. The fluid is sufficient to fill the space between coverslip and slide. Most of the red cells remain in the centre and the cell density becomes less towards the edges. Any agglutination is easily seen. After use, the coverslips are removed. The slides are rinsed under the tap, dried with a cloth, and are immediately ready for use again.

Sensitivity

Serial dilutions of anti-D sera have been incubated against Rh-positive cells, using this method parallel with the standard tube technique (Mollison, Mourant, and Race, 1948). Little difference in sensitivity has been found (see Table). With

TABLE

COMPARISON OF PRESENT METHOD AND STANDARD TUBE TECHNIQUE (MOLLISON, MOURANT, AND RACE, 1948)

Dilution of Test Serum	Present Method		Tube Method	
	Cell Sample A	Cell Sample B	Cell Sample A	Cell Sample B
1:1	+++	+++	+++	+++
1:2	++	+++	+++	+++
1:4	+++	+++	+++	++
1:8	++	++	++	+++
1:16	+	++	+	++
1:32	(+)	++	+	++
1:64	−	w	w	−
1:128	−	−	−	−

the present technique, cells are not drawn into and ejected from a pipette. Thus in a strong reaction almost all the cells are found stuck together in a continuous sheet.

The method is also suitable for ABO grouping, Rh genotyping, and titration of saline agglutinins.

I have to thank Mr. F. C. Pinion for his invaluable help in developing this technique and making the first set of apparatus. I am indebted to the Director-General, Army Medical Services, for permission to publish.

REFERENCE

Mollison, P. L., Mourant, A. E., and Race, R. L. (1948). *Medical Research Council Memorandum* No. 19. London: H.M. Stationery Office.

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A MULTIPLE MANUAL REGISTER FOR DIFFERENTIAL LEUCOCYTE COUNTS

BY

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One of the most trying and time-consuming operations in routine haematology is that of the differential leucocyte count. The method used in common practice is to write down on paper the different types of leucocytes as they are seen. To do so it is necessary for the person performing the count to be frequently looking away from his microscope. With a binocular microscope this routine can become very tiring, especially where many counts have to be made. With the monocular microscope also a great deal of time is lost. The multiple manual register was designed to overcome these difficulties. Experience with this tabulator has proved how valuable an asset it can be in a laboratory where many differential leucocyte counts have to be made, the time saved is considerable, and the general procedure of doing differential leucocyte counts has been eased to a most marked degree.

This instrument, which was produced by the Atomic Energy Research Establishment for the haematology department of the medical division, has been in use for several months. Because of the possible significance of the Arneth counts in cases of radiation exposure, the manual register was designed so as to allow for the differential leucocyte count and the Arneth count to be performed at the same time.

The instrument is designed to be operated by the left hand in order to leave the right hand free to operate the mechanical stage and fine focusing adjustment of the microscope. On this basis the lay-out of the different keys of the register has been arranged so as to place the keys of the leucocytes most commonly seen in the most convenient position for the operator. An even greater saving of time is effected if an assistant operates the register for the person doing the differential counts. It is a great help to the haematologist in studying the cytology of the blood film to be free of the necessity of interrupting his observations.

The multiple manual register differs from the "marbel blood cell calculator" which has been on the market for some time, in that the manual register possesses 13 different tabulator keys, compared with the eight keys numbered numerically, of the "marbel calculator"; furthermore the keys on the manual register are lettered for the different types of leucocytes commonly seen, and also with five numerals, 1 to 5, for the Arneth count (Fig. 1). These keys are easily detached by removing a small screw, and, as suggested by other workers, could be replaced with suitably indexed keys for bone marrow cell counts. The indicator lettering above each indi-

vidual register, corresponding to that on the appropriate key, may also be simply exchanged for any other form, representing a new set of indexed tabulator keys. The multiple manual register could be adopted for many purposes other than medical.

Mechanical Details of the Register

The multiple manual register (Fig. 1) incorporates 13 independent numerical registers. Each of these registers comprise two counting wheels (a units and a tens wheel) linked

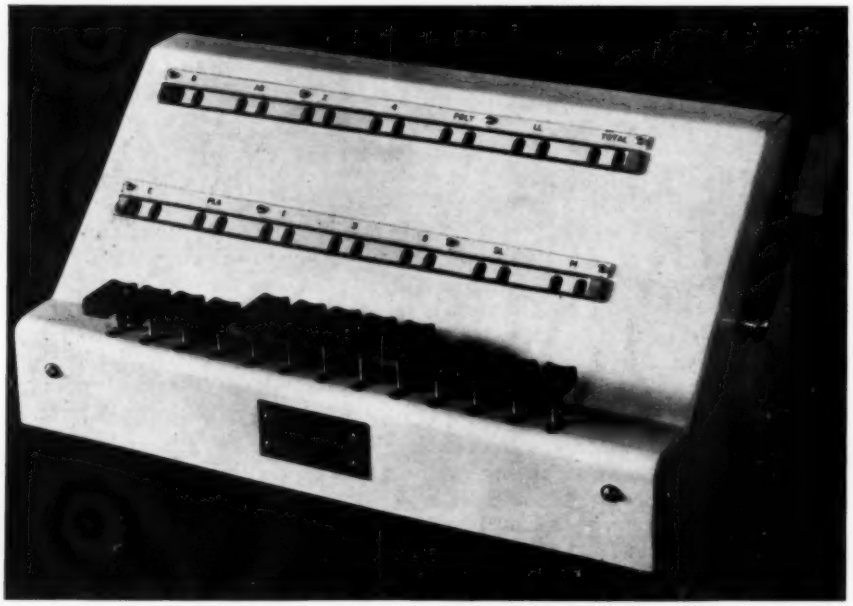

FIG. 1.—The register described.

E = Eosinophil. B = Basophil. PLA = Plasma Cell. AB = Abnormal Cell. 1, 2, 3, 4, 5 = Arneth, 1, 2, 3, 4, 5.
POLY = Polymorph. SL = Small Lymphocyte. LL = Large Lymphocyte. M = Monocyte.

together by a transfer pinion. This allows each register to record up to a maximum of 100 cells. The units drum of each register is coupled to a ten-toothed ratchet wheel, which is operated by a link mechanism from a key on the front of the instrument. The 13 keys are arranged in three groups of 4, 5, and 4 respectively, and are engraved (see key to Fig. 1) to represent eosinophil, basophil, plasma cell, abnormal cell (Arneth 1, 2, 3, 4, and 5), polymorph, small lymphocyte, large lymphocyte, and monocyte respectively. In addition to these registers there is a totalizing register operated by a bar linked to all 13 of the independent registers, which records a count each time any one of the 13 keys is pressed. It has two counting wheels and can therefore only record up to a total of 100. It is, however, coupled to a small bell in such a way that this bell is struck as the register changes from 99–00—i.e., every time a count of 100 is registered.

The complete set of registers may be reset to zero by the operation of a single lever mounted on the right-hand side of the instrument. To make the unit compact the various registers are assembled on two main spindles which are mounted so as to give a sloping faced unit which is easily read. The overall size of the register is 13 in. × 6½ in. × 6 in.

An improved model is being constructed which will allow the total Arneth count to be recorded independently of the overall total. This will be achieved by a second bar mechanism being fitted which will only be operated when any one of the five Arneth register keys are pressed. (These keys will still operate the main bar which covers all the 13 independent registers.) This subsidiary bar mechanism will be coupled to a second totalizing register consisting of two counting wheels—i.e., it will record up to 100 cells. It will also be fitted with a bell mechanism so that an alarm is given as this (the Arneth total) register changes from 99–00.

This new model will also have three instead of two counting wheels in the main totalizing register—i.e., it will record up to 1,000 cells. However, its alarm bell will be struck every hundredth count.

The reset action will be the same: one lever will reset to zero all the registers in the instrument.

Summary

A multiple manual register for differential leucocyte counts is described. The register incorporates 13 independent keys arranged in three groups of 4, 5, and 4 respectively, the middle five keys numerically lettered for the Arneth count. A bell signals each time that a total of 100 cells is registered. The adaptability of this register for other purposes than the differential leucocyte count has been suggested.

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English Numbering Machines, Ltd., have a development contract to engineer the improved model. The probable price is about £15. Owing to restrictions in dollar expenditure the "marbel blood cell calculator" is now unobtainable in this country.

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BLOOD PIPETTE SHAKING MACHINE

BY

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From the Medical and Engineering Divisions, Atomic Energy Research Establishment, Harwell

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The counting of red and white blood cells is probably one of the most frequent investigations performed in a routine haematological laboratory. The errors associated with this are well known (Biggs and Macmillan, 1948).

In this laboratory at least three pipettes are used for each patient as a routine, one red and two white cell pipettes. Each pipette is shaken for three minutes before the contents are placed on the haemacytometer for counting. This shaking time has been recommended as a minimum if reasonably accurate results are to be obtained (Macfarlane, 1938). The method employed here in the mixing of the contents of a pipette is to place the forefinger of the left hand over the tip of the pipette and with the right hand to rotate with a reciprocal motion, at the same time tilting the pipette to enable the glass bead to traverse the length of the chamber and around it. Therefore an apparatus was required that would: (1) reproduce this double motion of tilting and rotating; (2) enable up to six pipettes to be shaken at the same time; (3) hold pipettes of various lengths; (4) enable pipettes to be removed easily and quickly on completion of shaking; (5) be simple to operate, portable, mains-driven, and silent-running; (6) enable the period of shaking to be varied if required; (7) allow shaking process to be stopped at will; and (8) give a predetermined duration of shaking by control with an automatic process timer.

In any laboratory, such as ours, with a large number of counts daily, the time involved in shaking the pipettes is considerable. A mechanical method of blood pipette shaking would be of advantage in saving time, standardizing procedure, and possibly reducing variations due to incomplete mixing of the diluted blood in the pipettes.

As far as is known no machine capable of satisfying these requirements is available commercially, so one has been designed and made here. It is easy to use, gives no trouble, and is time-saving.

Description of Blood Pipette Shaking Machine

A small electric motor (a) drives through a coupling the single throw crank shaft (b) which engages with a slotted arm (c) attached to the shaft (d). Integral with the shaft is a toothed quadrant which engages with the pinion wheel (e). The pinion (e) is secured to the hollow spigot which is part of the pipette mounting body (f). Rotation of the motor output shaft imparts a tilting movement to the pipette mounting body about the axis "B"–"B" and rotating about the centre line "A"–"A" via the crank, slotted arm, toothed quadrant, and pinion.

Passing coaxially through the hollow spigot is a shaft (g) one end of which is permanently fixed to the mounting bracket (h); the other end carries a bevel wheel (j) terminating inside the pipette mounting body. A bevel wheel (k) carried on a shaft, mounted with and running coaxially inside the pipette mounting body, engages with the fixed bevel wheel (j). The other end of the shaft carries a larger gear wheel (l). Spaced around and engaging the gear wheel (l) are six pinions carried on shafts mounted in the pipette mounting body. The other end of each of these shafts terminates in a metal cup (m) housing a concave rubber seating. This seating carries one end of a pipette, the other end of which is carried in a spring loaded adjustable slide (n), fitted with a similar rubber seating, mounted on the pipette mounting body. This adjustable slide gives ease of insertion and detachment and enables varying lengths of pipette to be used. Six pipettes can be carried at the same time. The concave rubber seating prevents slipping and fluid loss.

Any tilting movement of the pipette mounting body imparts a rotary movement to the metal cup, via the pinion, gear (l), and bevel (k) as the latter rolls around the fixed pinion (j). Thus the motion imparted to the six pipettes is ± 30° tilt about the axis "B"–"B" and ± one and two-thirds revolutions per cycle. The total number of cycles per minute is approximately 90. The shaking time is controlled by a synchronous process timer. By means of knob (y) varying intervals from 0 to 5 minutes as required can be set on the timer. The timer is automatically reset at the end of a

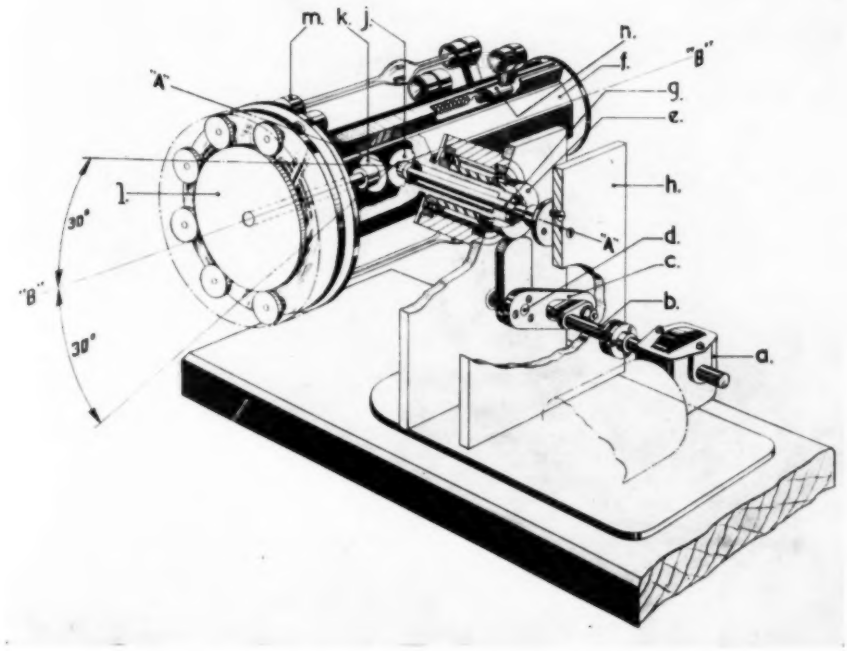

FIG. 1.—Diagram of the blood pipette shaking machine.

run so that each run takes the same time unless and until a different time interval is set on the dial. The shaking process can be stopped and automatically reset at any time by depressing button (x) and can be started by depressing button (t).

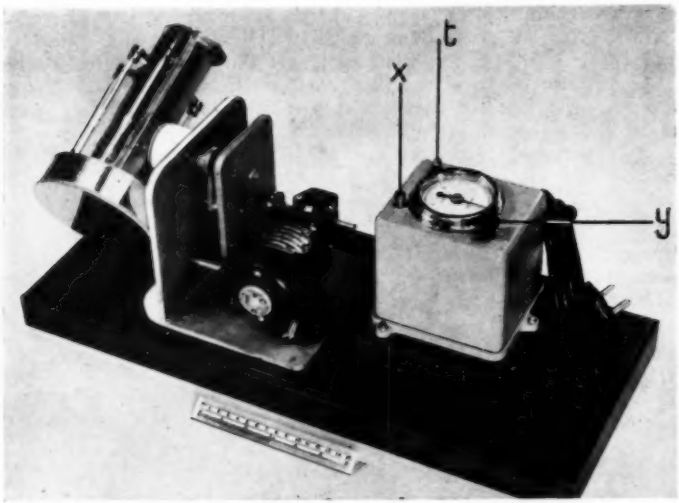

FIG. 2.—General view (cover removed).

Smooth, vibration-free running is obtained by a universal coupling between motor and main mechanism and all rotating shafts are carried in ball bearings. The mechanism, motor, and timer is housed in a dust-proof cover; all the rotating and moving parts on the pipette mounting body are enclosed. The pipette mounting body is nickel plated and polished for easy cleaning. The whole is mounted on a wooden base 22 in. long × 9 in. wide. The machine is portable. A mains supply of 230 volts 50 cycles is required for operation, and the apparatus can be plugged in to a 3 amp. plug.

A statistical survey is being carried out to compare the relative efficiency of hand-shaking with machine-shaking and to determine the minimum time for machine-shaking to ensure complete mixing.

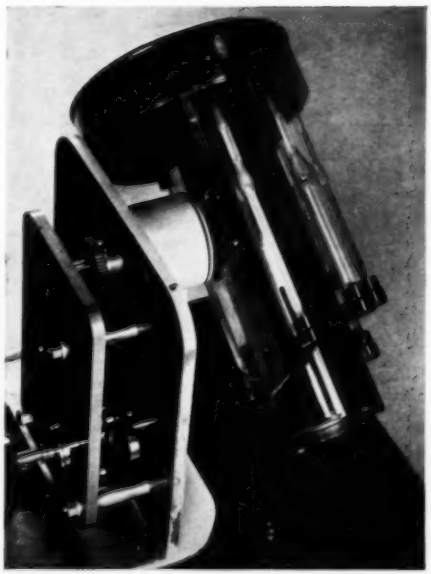

FIG. 3.—Tilting mechanism.

Should this machine be produced commercially the estimated cost is about £70, and, should the timing mechanism not be required, the cost would be proportionately less by about £10.

We would like to express our thanks to the Director, Atomic Energy Research Establishment, for permission to publish this article, also to Dr. Katharine Williams, principal medical officer, for her helpful criticism and encouragement, to Messrs. D. H. Oliver and A. W. Elson for their work in designing the machine, and to Mr. Brian Knight for the schematic drawing.

References

Biggs, R., and Macmillan, R. L. (1948). *J. clin. Path.*, **1**, 228.
Macfarlane, R. G. (1938). *Lancet*, **1**, 548.

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A MECHANICAL AID IN MAKING BLOOD FILMS

BY

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The apparatus which forms the subject of this paper was designed to eliminate some of the variable factors during the preparation of blood films by the slide method, and thus to reduce the errors of differential leucocyte counts. These errors can be grouped, according to their sources, into those arising during the preparation of the films due to a variety of technical factors, and into others inherent in every process of sampling, in which percentages of a total population are estimated from an analysis of small fractions. Efforts may justifiably be made to reduce the size of the first group of errors ; that of the second is irreducible.

The distribution of leucocytes on blood films is irregular, there being a relative excess of cells along the edges and at the end, an excess to which the various cell classes contribute to a varying extent: the larger a leucocyte, the more likely is it to be carried to the edges or the end (Gyllenswärd, 1929). Since lymphocytes are the smallest leucocytes, there is thus a relative lymphocytosis in the centre, and a relative lymphopenia at the edges of the film. MacGregor, Richards, and Loh (1940) have shown that a " battlement " count, which takes in parts of the edges and the adjacent interior, largely obviates this source of error, and gives results closely approximating those of counts of all leucocytes in the film.

Many factors, other than differences in cell size, may influence the quality of blood films and contribute to irregularities in the distribution of leucocytes. Thus the size of the drop of blood used, the speed of spreading, the width and nature of the spreading edge, the angle at which it is held, and the pressure applied, may all vary from observer to observer, and from occasion to occasion.‡ This problem has been analysed by Mainland, Coady, and Joseph (1936), who conclude that while it is impossible to incriminate any single factors, their combination leads to irregular spreading: " The variation . . . can be attributed to minor differences in cell size, and, perhaps, local variations in the current of the blood as it flows over the slide, which are uncontrollable factors."

The most important result of the irregular leucocyte distribution is the fact that a large number of cells have to be counted in order to ensure reasonable accuracy.

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‡Fundamentally the thickness of films and distribution of cells depend on local electrostatic and surface tension or capillary forces in relation to the wettability of the glass surface. If it were possible to obtain a surface, perfectly wettable by blood, the cell distribution should be entirely regular.

Thus Mainland, Coady, and Joseph (1936) have shown that, if three counts of 100 cells are done on the same films, none of them is equivalent to any of the others, even if no count includes cells from the " tail " of the film. It is usually recommended (e.g., by Beacom, 1928, and Medlar, 1931) that 300 or 400 cells should be counted as a routine. This figure might be expected to be reduced if the distribution of cells could be made more regular, so as to approximate to that of a binomial distribution. The sole remaining error would then be that inherent in sampling, and the size of the latter could be easily calculated (Goldner and Mann, 1938).

The Apparatus

The apparatus* permits the making of blood films on glass slides, keeping constant the width and consistence of the spreading edge, its angle with and pressure on the slide. This is achieved by moving the slide on a carriage under the pre-set and fixed spreader. A diagrammatic view is shown in Fig. 1.

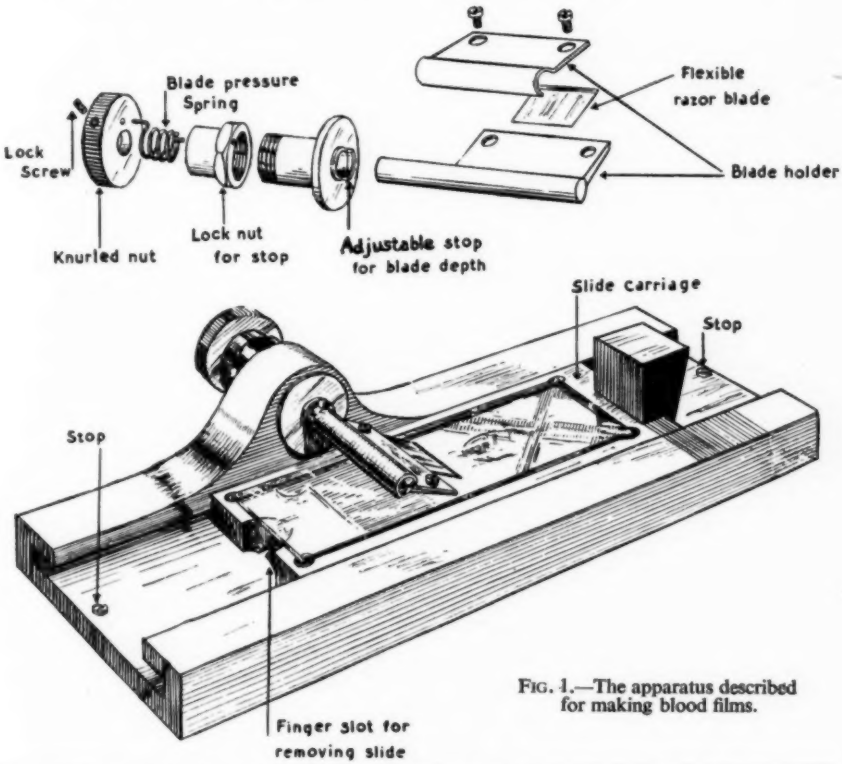

FIG. 1.—The apparatus described for making blood films.

* The original models of the apparatus were made in the Departments of Radiotherapeutics and Medicine, Cambridge University, by Mr. V. D. Martin and Mr. J. R. Flemans.

The spreader consists of a portion of a standard three-hole or slot razor blade and is held tightly in the metal blade holder. This blade holder is attached to a bar which runs through the slide support and has attached to its far end a knurled nut by means of which it can be rotated. Also attached to the blade is a stop which impinges on an adjustable stop on the side support. These stops fix the blade depth in relation to the slide. A weak spring is enclosed in the side support and attached to the knurled nut. It is adjustable for tension by rotation of the knurled nut, which in its turn is attached to the blade holder bar by a lock screw and provides the blade pressure on the slide.

The spreader is mounted on a side support on a troughed carriageway which has stops fitted at each end of the groove. Into this groove fits the slide carriage, which is recessed to hold the glass slide and has a knob at one end for easy traction.

The spreader holder is of necessity made of metal, but the carriage and carriageway can with advantage be made of "perspex," which provides a light, clean finished article.

The apparatus measures about 15 × 7½ × 4½ cm. and is easily portable.

Use of Apparatus.—A small drop of blood is placed at one end of a glass slide. This is best spread out in a thin line corresponding to the width of the blade. The slide is then placed in the slide carriage with the blood lying under the spreader holder. The carriage is drawn back until the stop is reached, thus causing the blood to form a thin film under the spreader edge. It is then moved with a steady slow movement in the opposite direction, and a thin film of blood is left on the slide. The thickness of this film can be varied by altering the amount of blood used and the speed with which the carriage is pushed. Provided the drop of blood used is small and the blade clean, the latter requires no cleaning after spreading, for no blood adheres to its edge. It should be renewed approximately once a month, when its edge becomes worn. A worn edge is shown by the presence of distortion and tearing of the cells along the edge of the film.

Films spread by this apparatus show absolutely straight edges, thus facilitating the performance of "battlement" counts. Their initial parts (about 1/3) tend to be thicker than those of hand-spread films, while the distal parts have the usual thickness and should be used for counting. The "tail" is often shorter than in hand-made films. The apparatus is easy to use, and even an inexperienced person can make satisfactory films with it after a very few trials.

Results

Two separate investigations were made in order to assess the value of the apparatus: (1) An analysis of cell distribution; (2) an investigation of cell size.

Analysis of Cell Distribution.—Venous blood from five normal individuals was used. After thorough shaking, 10 films were prepared from each blood by the same observer; of these five were made by hand, and five by the machine. There were thus available a total of 25 machine-made and 25 hand-made films.

Five differential counts were performed on each slide, employing the "battlement" procedure. Each single count was of 100 cells, classified into neutrophils, lymphocytes, monocytes, eosinophils, and basophils. In order to minimize personal bias which is liable to arise when the observer knows some of the previous results, all counts were recorded by an assistant, the observer remaining ignorant of the results until the end of the test. All counts were performed by the same individual.

The mathematical analysis given below has been made on the percentage of granulocytes present. A more refined analysis, which would deal with a suitable function of the percentage, was thought unnecessary, in view of the clear-cut results obtained. The aim was to compare the degree of homogeneity of the cell distribution in the hand-made and machine-made films.

For the purpose of analysis, we may first compare the cell distribution on a given slide with a binomial one, by means of Pearson's χ^2 test. For each slide we have five counts, each distinguishing two classes: granulocytes and the rest; this will give a χ^2 with four degrees of freedom. We can combine these for the five slides of each of the five bloods to give a composite χ^2 with 100 degrees of freedom. The values obtained are:

Hand method $\chi^2_{100} = 221.7$ $P < 0.1\%$
Machine method $\chi^2_{100} = 121.7$ $P = 6.8\%$

The value for the hand method is highly significant, and hence the cell distribution in hand-made films shows a highly significant departure from a binomial one. The distribution in machine-made films, on the other hand, shows no significant departure from a binomial one, accepting the customary 5% level of significance.

If we now analyse the total counts of 500 cells for each slide, we can calculate for each of the five bloods a χ^2 with four degrees of freedom. Adding the quantities for the different bloods gives a χ^2 with 20 degrees of freedom. The results are:

Hand method $\chi^2_{20} = 23.6$
Machine method $\chi^2_{20} = 20.3$

Neither value is significant.

This analysis shows that as far as the proportion of granulocytes is concerned, there is a binomial distribution for both methods of spreading, if we consider large counts of the order of 500 taken from each slide. The data from different slides appear to be homogeneous, and we can calculate the standard errors in the usual way. On the other hand, if we look at several counts of 100 cells drawn from a single slide, then while there is probably still a binomial distribution in the machine-made films, this is definitely not so in films spread by the old method.

It is possible to examine the same data in a different way, by analysing the degree of irregularity in results due to variation between slides and within slides. This is done in the analysis of variance in Table I, which shows that with the machine

TABLE I

ANALYSIS OF VARIANCE OF PERCENTAGE OF GRANULOCYTES IN 125 COUNTS OF 100 CELLS IN HAND-MADE AND 125 IN MACHINE-MADE FILMS

Source of Variation	Old Method			New Method		
	Sum of Sq.	D. of F.	Mean Sq.	Sum of Sq.	D. of F.	Mean Sq.
Within slides ..	5026.40	100	50.26	2711.20	100	27.11
Between slides ..	509.68	20	25.48	463.04	20	23.15
Between bloods ..	2364.85	4	591.21	1720.05	4	430.01
Total ..	7900.93	124		4894.29	124	

method the degree of variation within slides does not differ significantly from that between different slides. But with the old method the variation within slides is twice as great as the variation between slides: the difference is significant, $P < 5\%$.

Thus if we employed single counts of 100 the variation would be approximately twice as great using the old method of spreading. It is pertinent to ask whether this disadvantage is overcome before reaching the count-size of 500, at which, as we have seen above, the distribution can be taken to be binomial. In order to answer this question, the first two counts on each slide were selected and added together, making a total count of 200. The variation of these counts about the blood means was then examined and the variance was found to be approximately 77 compared with the 54 expected from the new method. Thus for counts of 200 the old method has about 70% of the efficiency of the new method. Counts of 300, 400, and 500 showed no difference between the methods.

The advantage of machine-spread slides is therefore that we can assume a binomial distribution for leucocyte counts as low as 100, and thus regard such counts as representative of the overall distribution on the slide; further, the variation within single slides and between a series of films of the same blood is about the same, so that a differential count of 100 leucocytes is representative of the whole series and can be used for calculating the remaining statistical error and establishing confidence limits. With hand-made films, counts of 100 or 200 cells are respectively only 50% and 70% as accurate as counts of the same size on machine-made films; if 300 cells are counted, no difference is found between the two methods. The analysis of films made by hand confirms the findings of Mainland, Coady, and Joseph (1936), and also supplies new evidence in support of the frequently voiced opinion that at least 300 cells should be counted to obtain a reasonably accurate result in routine counting.

Investigation of Cell Size—The size of leucocytes is occasionally measured in blood films, and it is reasonable to assume that the values found depend partly on the amount of distortion of individual cells introduced by the process of spreading. The aim of this investigation was to compare the degree of distortion in hand-made and machine-made films.

Normal venous blood was obtained from the same individual on two different occasions. On the first, five films were made by machine and four by hand; on the second four films by machine and four by hand. The hand-made films were prepared by different skilled workers on the two occasions. There were thus available nine machine-made and eight hand-made films, the latter subdivided into two groups of four, made respectively by workers W_1 and W_2.

On each film, the size of 100 consecutive neutrophils was determined by means of a micrometer eyepiece, the mean of two readings at right angles being expressed in arbitrary scale divisions. In all films cells were measured in the same order, by moving from one edge to the other across the middle of the films, until the full 100 cells had been included.

The data analysed are the mean and standard deviation for each slide. These are given in Table II.

We can employ Student's t-test to test the significance of the differences between the means of the various groups in which we are interested, using an estimate of

TABLE II

SIZES OF NEUTROPHILS COMPARED IN MACHINE- AND HAND-MADE FILMS

	Type	Slide	Mean	Standard Deviation
Machine-made	T_1*	M_1	17.55	1.23
		M_2	17.86	1.01
		M_3	17.76	1.42
		M_4	18.35	1.34
		M_5	18.28	1.33
	Average T_1	=	17.96	1.27
	T_2	M_6	17.70	1.14
		M_7	18.34	1.12
		M_8	17.45	1.14
		M_9	17.48	1.02
	Average T_2	=	17.74	1.10
Hand-made	W_1	H_1	19.13	1.51
		H_2	18.84	1.49
		H_3	20.04	1.24
		H_4	18.88	1.41
	Average W_1	=	19.22	1.41
	W_2	H_5	18.47	1.21
		H_6	19.15	1.17
		H_7	19.76	1.22
		H_8	19.03	1.01
	Average W_2	=	19.10	1.15

* Slides T_1 and W_1 made from one sample of blood, slides T_2 and W_2 from a later sample of the same blood.

variance based on the total of 13 degrees of freedom within the four main classes—i.e., four from T_1 and three from each of T_2, W_1, and W_2.

Among the means the only difference which is significant is that between the averages for hand-made and machine-made—i.e., $19.16 - 17.86 = 1.30$. The standard error of the difference under consideration is 0.224. This makes $t = 5.80$; with 13 degrees of freedom this is significant with $P < 0.1\%$.

Among the standard deviations, again only one difference is significant—that between the averages for the two operators—i.e., $1.41 - 1.15 = 0.26$. The corresponding standard error is 0.084, and thus $t = 3.10$; with 13 degrees of freedom this is significant with $P < 1\%$. The value of t for the difference between the averages for the two machine-made groups is 2.02, for which $P = 6.4\%$: the difference, though substantial, is not significant.

The analysis as a whole suggests that the mean size of 100 cells from a given slide is somewhat smaller when the slide is machine-made. There is no noticeable difference in mean size between the two machine-made groups or between the two hand-made groups.

So far as variation in size goes, the difference between the two machine-made groups is not significant at 5%, though the actual value of P, 6.4%, suggests a possible

difference. The difference between the two hand-made groups is significant, however, with $P<1\%$. Thus the degree of distortion of leucocytes produced in the machine-spread films is more uniform than that in hand-made ones, if the latter are made by more than one observer. When therefore variations in cell size between different films have to be determined, it is advantageous to use the machine for spreading the blood, as the degree of error introduced by accidental technical factors is thereby diminished.

Summary and Conclusions

The paper describes an apparatus designed to eliminate some accidental factors contributing to irregularity in the composition of blood films made on slides. With its aid good films can be produced with very little practice, even by inexperienced workers. Films are characterized by very straight edges, facilitating "battlement" counting, and a relatively short "tail."

An analysis of differential counts performed on hand-made and machine-made films confirmed the previously known fact that in the former, owing to irregularities in the cell distribution, at least 300 cells have to be counted in order to obtain results representative of the total count in the film, or in a series of films made from the same sample of blood. In machine-made films, on the other hand, the distribution was found to resemble a binomial one, for counts as low as 100 leucocytes, thus allowing the easy calculation of the statistical error and of confidence limits. The use of this apparatus saves considerable time in routine differential counting and gives an increase of accuracy for counts of less than 300 leucocytes.

A comparison of the size of leucocytes showed a smaller mean size in machine-made than in hand-made films, and a greater variation in cell size between two groups of slides made by hand by two different observers, than between two corresponding groups made by machine. The apparatus thus produces a more uniform cell distortion than the usual hand method, a fact of some importance where sizes of leucocytes are to be compared in different films.

The authors gratefully acknowledge the valuable advice on design of the apparatus given to them by Mr. D. J. Allen-Williams, M.A., A.M.Inst.C.E., A.M.I.E.E., of the Department of Radiotherapeutics. The apparatus is now being made by the English Instrument Company, Mill Lane, Cambridge. They also wish to thank Professor Sir Lionel Whitby and Professor J. Mitchell, in whose department the work was performed, for their interest.

References

Beacom, D. N. (1928). *J. Lab. clin. Med.*, **13**, 366.
Goldner, F. M., and Mann, W. N. (1938). *Guy's Hosp. Rep.*, **88**, 54.
Gyllenswärd, C. (1929). *Acta paediatr., Stockh.*, **8**, Suppl. 2.
MacGregor, R. G. S., Richards, W., and Loh, G. L. (1940). *J. Path. Bact.*, **51**, 337.
Mainland, D., Coady, B. K., and Joseph, S. (1936). *Folia Haemat., Lpz.*, **54**, 8.
Medlar, E. M. (1931). *J. Lab. clin. Med.*, **17**, 169.

J. clin. Path. (1950), 3, 175.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 43rd SCIENTIFIC MEETING

The forty-third scientific meeting of the Association of Clinical Pathologists was held in the Royal Pavilion, Brighton, from January 26 to 28, 1950, under the Presidency of Dr. Norah Schuster.

At the first session papers were read by J. Ungar (Greenford), R. Cruickshank (London), A. Macdonald (Liverpool), and G. Stewart Smith (Exeter). Summaries of these papers follow.

Comparison of Penicillin Levels in Blood and Inflamed Tissues

Dr. UNGAR said that experiments with rabbits, in which artificial muscular oedema was caused by injection of turpentine, showed that after a single intramuscular injection of penicillin, the latter could be detected in the tissue exudate for considerably longer periods than in the blood. Levels of penicillin were demonstrable in concentrations and after lengths of time sufficient for effective antibacterial action, but no penicillin was detected in the organs of rabbits three or five hours after injection of a single dose of penicillin. This selective accumulation of penicillin in tissue exudate was one of the important factors responsible for the therapeutic effect of single massive doses of penicillin, and gave further justification for this method of treatment, involving less frequent injections. The accumulation of penicillin in the exudate was noticed with aqueous solutions of either crystalline or amorphous penicillin, of penicillin in oil–beeswax suspensions, or of procaine penicillin suspensions. Tissue was shown injected with penicillins G, F, and K.

The Present Position of the Newer Antibiotics

Professor ROBERT CRUICKSHANK spoke about the present position of the newer antibiotics. Besides the infections scheduled by the Ministry of Health as being suitable for treatment with streptomycin and/or chloramphenicol, the Medical Research Council's Antibiotics Trials (Non-tuberculous Infections) Committee was testing these drugs in a number of other infections, including whooping-cough, in different centres in London and the provinces.

With the increasing number of antibiotics now becoming available, the clinician was more often seeking the help of the clinical pathologist not only in regard to the drug sensitivity of the pathogen, but also on the choice of drug and the dosage for particular infections. Factors to be considered in this connexion were the efficiency of the antibiotic, *in vivo* as well as *in vitro*; the risk of toxic effects; and the ease and cost of administration. The variety of causative agents in such conditions as pneumonia, meningitis, and urinary infections called for careful laboratory investigation to establish the drug of choice. More work was needed on the value of combined therapy. Certain drugs have a synergistic action, and the combination of another bacteriostatic drug with streptomycin may inhibit the emergence of streptomycin-resistant pathogens as has happened in the treatment of pulmonary tuberculosis with streptomycin and P.A.S. He advocated the use of massive dosage of penicillin in subacute bacterial endocarditis and in staphylococcal septicaemia, even when the organism was apparently penicillin-resistant. He showed the *in vitro* sensitivity of a wide range of bacterial pathogens to aureomycin, chloramphenicol, and polymyxin, discussed the relative merits of these new drugs, and indicated the methods which might be used for testing bacterial sensitivity and for assay of the drug-levels in blood and other body fluids.

Laboratory Diagnosis of *Toxoplasma* Infection

Dr. MACDONALD spoke of the value of the various laboratory procedures used in the diagnosis of toxoplasmosis, and said that the complement fixation test was probably the most convenient for the ordinary laboratory. Sabin and Feldman's test for cytoplasm-modifying antibody was extremely sensitive, but required the use of living parasites. The soluble egg antigen used for complement fixation tests maintained its potency if kept in the frozen state. In Liverpool they had found good general agreement between the results of different types of test. Examinations of maternal and cord blood showed that transference of maternal antibody occurred so that serological diagnosis had to be made with caution in very young infants. Dr. Macdonald also discussed his serological findings in children with various clinical signs. While he had obtained evidence that infection with *Toxoplasma* was not so rare as had been thought, it was well to remember that toxoplasmosis was only one of a number of conditions which could produce very similar clinical manifestations.

Pulmonary Tuberculosis as a Necropsy Hazard

Dr. STEWART SMITH briefly reviewed the literature in the American medical press which indicated that in three different universities in America and in one in Sweden it had been noted that medical students were more liable to develop tuberculosis than those in other faculties, and that a significant increase in cases occurred while the students were attending necropsies, there a pre-clinical subject. Thus, possible sources of infection were narrowed down. Experimental evidence showed that tubercle bacilli were disseminated from fresh tuberculous material and could be grown from towels, instruments, and gloves for 24 hours after a necropsy. A rigorous review of necropsy-room hygiene had in each case reduced the risk.

Dr. Stewart Smith said that during the past 25 years he knew of six necropsy-room attendants who had developed pulmonary tuberculosis, and three of them had died from the disease. The speaker then outlined measures which could be undertaken if the experience of other members of the Association confirmed that there was an appreciable hazard of pulmonary tuberculosis associated with necropsies. These were (1) a review of autopsy-room hygiene, (2) Mantoux tests and periodic radiological examination of staff, (3) scheduling tuberculosis as an industrial hazard of necropsy work, and (4) limitation of the number of necropsies on frank tuberculous cases.

At the morning session on January 27, the President, Dr. Norah Schuster, took the chair, and papers were read by A. Richardson-Jones (Portsmouth), C. D. Anderson and G. B. Roberts (Glasgow), D. F. Cappell and J. R. Anderson (Glasgow), and A. Spriggs (Oxford). These are summarized below.

Dextran as a Potentiator of Incomplete Anti-Rh Antibodies

Dr. RICHARDSON-JONES said that dextran had shown itself to possess a potentiating action, but in the state in which it was commercially available was unsatisfactory for routine laboratory use because it caused gross rouleaux formation of the test cells and failed to potentiate all incomplete antibodies. He outlined the factors which he considered to contribute to rouleaux formation with dextran : the age of the test cell and the resistance to rouleaux possessed by unwashed cells.

The failure of commercial dextran to potentiate all incomplete sera was thought to be related to its polydisperse constitution. The potentiating property was due to a relatively narrow range of molecular size, and furthermore it seemed as though certain other molecules had an inhibitory effect. In an attempt to relate potentiating power to molecular size he had undertaken the acetone fractionation of commercial dextran. Although this phase of the work was incomplete, he had succeeded in producing a fraction which potentiated all the incomplete anti-sera with which it had been tested. It caused no rouleaux, and gave antibody titres closely comparable with those obtained by albumin potentiation. He felt that this fraction offered a substitute for bovine albumin in cross matching for transfusion and in testing the serum of pregnant women for anti-Rh antibodies.

Dextran was chemically stable and cheap.

Acute Monocytic Leukaemia Terminating with Multiple Chloromata

Drs. ANDERSON and ROBERTS reported a case of monocytic leukaemia in a young girl who had had a leukaemic blood picture and a highly cellular leukaemic marrow.

The patient, after treatment with urethane, developed staphylococcal pneumonia, successfully treated with penicillin. Subsequently, the white cell count in the peripheral blood fell to normal and mature cells reappeared. The patient was discharged, but three weeks later lumps appeared in both breasts. One lump was removed and found to be a chloroma, the histological features of which were very similar to those seen in the bone marrow on admission. Shortly afterwards the patient developed paraplegia and died. Localized chloromatous tumours were found in breast and meninges, both on the surface of the brain and spinal cord. There was no evidence of a diffuse leukaemic process. The bone marrow was normal, and the spleen showed old healed infarcts. The termination of the diffuse leukaemic process had been regarded as an apparent cure. The development of frank leukaemia in chloroma was considered to be a frequent terminal event, but the subsequent development of chloromata in apparently cured leukaemia was unusual.

Mothball Anaemia

Professor D. F. CAPPELL and Dr. J. R. ANDERSON presented a case of megalocytic anaemia with hyperplastic marrow of megaloblastic type. The patient was a girl of 17 whose history included mental disturbances and persistent gastro-intestinal upset. On admission to hospital she was critically ill with a profound anaemia. The findings in examinations of the peripheral blood and sternal marrow were indistinguishable from true pernicious anaemia in relapse. The gastric contents contained free HCl. In spite of slow drip blood transfusion and liver therapy the patient died after three days in hospital.

Necropsy showed the general changes of severe megalocytic anaemia, but without evidence of atrophy of the gastric mucosa. The ileum showed an unusual inflammatory lesion, most severe in its mid-portion where the mucosa was partially necrotic, and incorporated in a thick firmly adherent membrane which resembled fibrin, but was shown microscopically to consist largely of inspissated mucin. Search for a history of possible consumption of drugs revealed that the girl had been in the habit of sucking naphthalene mothballs. Some of the reported cases of acute haemolytic anaemia resultant upon ingestion of naphthalene were mentioned; chronic naphthalene poisoning was noted to be rare, and no record of a chronic anaemia or of a fatal case of chronic poisoning could be found. The metabolism of naphthalene in experimental animals was briefly described.

It was concluded that the case was a true megalocytic anaemia, such as results from deficiency of the haemopoietic principle. None of the usual causes of such an anaemia appeared applicable, and it was suggested that either (1) the prolonged absorption of naphthalene had produced a chronic haemolytic anaemia, which, in association with impairment of absorption consequent upon the intestinal lesion, might have resulted in exhaustion of the haemopoietic principle, or (2) naphthalene may have some more direct inhibitory or destructive action upon the haemopoietic principle or its component factors.

The Morphology of Cells in Pleural and Peritoneal Exudates

Dr. SPRIGGS showed a number of colour photomicrographs illustrating the morphology of the cells of pleural and peritoneal effusions. He described the technique of preparing the smears, a thin, quick-drying film being essential to success. The smears were stained by May–Grünewald–Giemsa and other methods; for instance, mucus secretion had been demonstrated in malignant cells of carcinomatous ascites by the periodic-acid-Schiff technique (following salivary digestion to exclude glycogen).

Dr. Spriggs said that 255 fluids from 175 cases had been examined. Of 30 effusions proved malignant by biopsy of a growth, laparotomy, or necropsy, 20 contained recognizable malignant cells.

The morphology of the endothelial cells was illustrated for comparison. Although frequently showing multinucleate forms and

mitotic figures, the round or oval nuclei showed little variation. Abundant mitotic figures had been found in those effusions where plasma cells were frequent. Slides were also shown illustrating the degeneration of neutrophils in sterile and in infected fluids, the phagocytic properties of macrophages, and the occurrence of unusual cell types, such as "Mott cells," promyelocytes, and megaloblasts.

The second group of papers read on January 27 were by N. F. Maclagan and Barbara Lunnon (London), M. A. Bundock and J. N. Cumings (London), and C. Dent (London), and are summarized.

After lunch two films, by courtesy of the Central Office of Information, were shown: (1) *Tracing the Spread of Infection in Pemphigus Neonatorum*, and (2) *Another Case of Food Poisoning*.

Flocculation Tests

Professor MACLAGAN and Miss BARBARA LUNNON gave an account of their results with the zinc sulphate turbidity test which had been correlated with the serum globulin level and with other flocculation tests in a series of 100 cases in which the diagnosis was reasonably certain on clinical grounds.

They found a less close correlation with serum globulin than that described by Kunkel, but a very close correlation with the serum colloidal gold test. The zinc sulphate test was also roughly correlated with the thymol test, but there were considerable differences in the number of cases. It appeared that the zinc sulphate test was probably more sensitive in chronic hepatitis than the thymol test, but on the other hand it was more likely to give positive results in non-hepatic conditions. The thymol test appeared to be more sensitive in acute hepatitis and gave occasional false positive results in cases of lipaemia.

While the zinc sulphate and thymol tests were frequently in agreement, useful information was sometimes obtained only by one of these tests in particular cases. It was therefore recommended that for diagnostic purposes the best results would be obtained by combining the two tests.

Methods for Estimating the Lange Reaction in Cerebrospinal Fluids

Drs. BUNDOCK and CUMINGS gave an account of a comparison they had made between three methods of performing the Lange colloidal gold test on cerebrospinal fluids from various diseases, but with special reference to neurosyphilis. The techniques used were (1) the standard citrate gold method with saline dilutions of C.S.F.; (2) Maclagan's six tube method with buffered dilutions of C.S.F.; and (3) Lange's method of 1939 in which gum ghatti is used to stop the reaction of a citrate gold with buffered dilutions of C.S.F., and in which specially prepared standards are used for colour matching.

Reasonably good agreement between all three methods was demonstrated in normal fluids and in those fluids showing only minimal chemical abnormalities. The first method was shown to give a few midzone curves, but these gave rise to no difficulty in interpretation when considered with the other findings. It was shown to parallel the clinical findings in general paralysis and tabes better than the third method, and both the first and third methods better than the second. Tables showing the results found in other diseases were also shown, and some variation between the results in the three methods was demonstrated.

The third method was considered to be technically rather difficult, and although useful for a laboratory dealing with many C.S.F.s daily, it was not recommended for routine use in an ordinary clinical laboratory. Drs. Bundock and Cumings recommended the first method, the standard technique, which gave most help to the clinician and was reasonably accurate and relatively simple to perform.

The Technique and Scope of Paper Partition Chromotography

Dr. DENT began by describing the discovery in 1944 by Consden, Gordon, and Martin of a revolutionary method of qualitative analysis. Only filter paper, a few solvents, and a minimum of apparatus were required, and although originally devised for amino-acids in fairly pure solutions, it was now finding applications with many other types of compounds,

for instance, sugars. Furthermore it could be used with very crude mixtures of partly unknown composition such as urine, plasma, sweat, cerebrospinal fluid, tissue extracts, etc.

There were various techniques. In the most simple form strips (2 × 50 cm.) of filter paper were used, the urine (or other fluid to be analysed) being placed near one end, allowed to dry, the end being then dipped into a vessel containing the solvent (usually water-saturated phenol). When the solvent had soaked along the strip a suitable distance it was dried off in an oven. The strip was then sprayed with ninhydrin solution and heated to 100° C. for a few minutes. The amino-acids could now be seen in the form of purple bands spread along the strip where the solvent had passed, and could be provisionally identified from the position they had taken along the strip. A more accurate identification was obtained if large squares (18 × 22 in.) of filter paper were used, two solvents being successively soaked along the paper in directions at right angles to each other. A rapid, mass production method using smaller squares had now been developed. The papers were held together in a frame and almost any number could be run simultaneously. Dr. Dent thought that this should be the most suitable method for routine use.

The method was, first, an ultra-micro method, only microgram quantities of materials being required. As a result, only a few drops of, for instance, urine, were necessary for a fairly complete analysis. Secondly, it was eminently suitable for the study of the metabolism of radioactive materials, since the presence of these on the paper chromatograms could be revealed by pressing the paper against an *x*-ray plate. Thirdly, although it was essentially a qualitative method, it was nevertheless likely to be developed further so that accurate quantitative work could be done.

It must be emphasized, however, that its most spectacular value at the moment was as a purely research weapon. With regard to its ordinary medical applications it was so far limited to the diagnosis of a few rare metabolic disorders in the amino-acid field, and to the identification of sugars and porphyrins. There was no further need to search for cystine and tyrosine crystals in urine. Dr. Dent nevertheless believed that at the present pace of development many other substances, such as steroids, would soon come within the scope of the method and that it was therefore only a matter of time before most laboratories would find it necessary to have some sort of paper chromatographic apparatus.

The Fanconi Syndrome and Other Problems of Amino-acid Metabolism

Dr. DENT stated that in 1936 Fanconi described three cases of an unusual form of "renal rickets." They were all in young children who presented with gastro-intestinal disorders, dwarfism, and the typical bony deformities and *x*-ray signs of florid rickets. The bone condition appeared to be unaffected by the usual doses of vitamin D. In addition, the cases showed a chronic acidosis, proteinuria, renal glycosuria, and an inability to pass an acid urine. However, unlike the findings in classical renal rickets the blood urea and NPN were normal or only slightly raised and the serum inorganic phosphate was low. Other curious urinary features were a high titration for organic acids, a high ammonia coefficient, and some evidence that crystals resembling leucine could be obtained on concentration of the urine.

Similar cases were described about the same time by Debré and by de Toni and later workers showed that the disease was a true entity. More recently an apparently identical condition, in a much more chronic form, had been found in adults, which presented, usually in middle age, as a typical osteomalacia. There was some difficulty in interpreting the literature dealing with a related condition, cystinosis, in which large cystine deposits had been found in the reticulo-endothelial system.

Dr. Dent had been able to collect records and urine specimens from at least 18 cases of the Fanconi syndrome. Chromatographic analysis readily confirmed that there was a gross amino-aciduria involving most of the common amino-acids. Chemical and chromatographic analysis of the blood in several cases showed, however, that the plasma amino-acids were quite normal in their concentrations. The excretion must therefore by definition have been the result of a low renal threshold due to defective tubular reabsorption mechanisms. Other tubular functions were also defective, namely the glucose and phosphate reabsorption, the urine acidification mechanism, and, in some cases, there was poor water reabsorption, which showed clinically as a pitressin-resistant polyuria.

The excretion of large amounts of many amino-acids in the urine was, according to the

previous literature, an excessively rare occurrence, only occurring with regularity in acute yellow atrophy of the liver in which the onset of the amino-aciduria coincided with, and presumably resulted from, a large rise in the blood levels of amino-acids, an "overflow mechanism." Dr. Dent had confirmed chromatographically these hypotheses in five cases of acute yellow atrophy. Further work had now shown that the "overflow mechanism" occurred also in phenylketonuria, where, however, only one amino-acid, phenylalanine, was involved, and when a rapid intravenous transfusion of protein hydrolysate was given, large quantities appearing in the urine. The renal mechanism was the more common, occurring not only in the Fanconi syndrome but also in hepatolenticular degeneration and in cystinuria. There were also interesting variations, relatable to tubule function, in the patterns of excretion of amino-acids in normal people. Most of these renal mechanisms seemed to be under direct genetic control, but acquired tubule damage could cause a similar picture as, for instance, after lead or uranium poisoning. Dr. Dent thought that the term "error of metabolism" when applied as an explanation of amino-aciduria, should be restricted to mechanisms of the "overflow" type where the body tissues as a whole seemed to be unable to metabolize certain substances at their normal reaction rates. He had studied seven cases of cystinuria and had found no clinical or chemical evidence to suggest that this was anything more than a renal cystinuria. It seemed no more justifiable to describe it as an "error of metabolism" of an amino-acid, than it would be to describe the well-known condition of renal glycosuria as an error of glucose metabolism. The question now was why this usually inherited condition of renal amino-aciduria could be associated in some cases with no ascertainable disease at all, and in other cases with such serious organic diseases so utterly unrelated to each other clinically as the Fanconi syndrome and hepatolenticular degeneration. Dr. Dent said that he could not account for this curious anomaly, but could only take advantage of the excellent diagnostic facilities offered by the amino-aciduria.

After tea a paper was read by F. T. G. Prunty (London). Dr. Prunty's paper, "Laboratory Aids in the Assessment of Adrenal Cortical Function," is published in full on page 87 of this issue. Professor G. W. Pickering (London) and Professor Dorothy Russell (London) opened a discussion on aspects of benign and malignant hypertension.

The papers on January 28 were read by C. W. Taylor (Birmingham), N. H. Ashton (London), K. S. Thompson (Birmingham), and G. R. Osborn (Derby), and summaries follow.

Fatal Maternal Embolism by Amniotic Fluid Contents

Dr. C. W. TAYLOR demonstrated the findings from a case of fatal maternal embolism due to amniotic fluid contents, and stressed the importance of microscopic examination of the lungs in patients dying either during or soon after delivery of apparent obstetric shock.

The main clinical features are usually those of a healthy multigravida with a large foetus, very strong uterine contractions, and stillborn baby. During labour or shortly after delivery there is a sudden onset of profound shock with cyanosis and dyspnoea terminating fatally in a few minutes or, at the most, a few hours.

At necropsy there is usually no obvious cause of death, and the emboli are only seen on microscopy.

In the case described, emboli were present in the brain and heart muscle as well as in the lungs. The emboli consisted of plugs of epithelial squames similar to those seen in the alveoli of infants who have attempted to breathe *in utero*.

New Techniques in the Laboratory Examination of the Eye

Dr. NORMAN ASHTON described new methods which had been devised and familiar techniques which had only recently been applied in the research and routine examination of the eye. He showed how the retina might be examined macroscopically and microscopically by mounting it in a glass sphere the same size as the sclera, and retinal micro-aneurysms demonstrated by benzidene staining; by the periodic method of McManus; by indian ink; and by neoprene injection of the central retinal vessels. Dr. Ashton showed neoprene casts of

Schlemm's canal and its branches, and pointed out the value of this technique in the study of aqueous veins. He described his museum mounting techniques, particularly those using solid "perspex" and "marco" resin. He concluded with an account of the examination of the aqueous humour by puncture of the anterior chamber, and spoke of his experiences with the "hot" celloidin embedding technique for rapid section of the whole eye.

A Correlation of the Histology and Cytology of the Female Genital Tract

Dr. G. R. OSBORN gave an account of his experiences with the Papanicolaou method of cytodiagnosis applied to the female genital tract.

The Papanicolaou smear was at its best in cases of carcinoma *in situ* and typical squamous-cell carcinoma of the cervix. Some tumours, especially adenoacanthomata of the endocervix and recurrences after radium treatment, had been found to desquamate few or no cells, although studied repeatedly. It was because of the existence of this type of tumour that smears of cervical scrapings should be examined. The surgeon had to be skilled technically or the smears were hopeless for diagnosis.

Adenocarcinoma of the body of the uterus often shed actual tumour fragments containing mitotic figures, as a rule fully diagnostic, as they drew attention to the importance of seedling implants in the Fallopian tubes, endocervix, vagina, and operation wounds.

The most important pitfalls in cytodiagnosis were badly taken and badly stained material; trichomonas vaginalis, which could be recognized with experience to give pathological smears unlikely to be confused with cancer; degenerating epithelial cells (especially in atrophic vaginitis) which should not be confused with mitotic figures; atrophic vaginitis, responding to oestrogens; histiocytes, which could cause endless mistakes, as with clusters of endometrial cells they gave false positive diagnoses; inflammatory reactions giving very bizarre appearances; the carcinoma without desquamation would be missed by the ordinary methods, a negative report not necessarily indicating the absence of a tumour; and the carcinoma which desquamated so few cells that a confident diagnosis was not made.

Dr. Osborn did not think that the value of the Papanicolaou smear in the diagnosis of radiation response was proved.

The meeting ended with a discussion on "The Reconstruction of Human Remains" which was opened by Professor J. D. Boyd (London), and those taking part included C. Keither Simpson (London), R. D. Teare (London), F. D. M. Hocking (Truro), and D. E. Price (Barnsley).

About 200 members and guests attended the meeting. The guests included Dr. D. Jaumain (Belgium) and Professor Raoul Kourilsky (Paris), president of the Societé Française du Biologie Clinique.

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ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Clotting of Human Citrated Plasma by Gram-negative Organisms. HARPER, E. M., and CONWAY, N. S. (1948). *J. Path. Bact.*, **60**, 247.

Many Gram-negative bacteria have been found to be capable of coagulating citrated plasma. This they do by metabolizing the citrate and thus liberating the free calcium ions required for normal plasma clotting. In oxalated plasma and in much-diluted citrated plasma no coagulation occurs. The nature of the coagulation effected by these organisms is thus basically different from that initiated by the coagulase-positive strains of *Staphylococcus aureus*. The two kinds of clotting can be distinguished by the addition of heparin to the citrated plasma; this renders it no longer coagulable by the Gram-negative bacteria, though it can still be clotted by active strains of staphylococci.

G. Payling Wright.

The Effect of Caronamide on the Blood Concentration of Penicillin Following Oral and Intramuscular Administration of Penicillin. ZELLER, W. W., LEPPER, M. H., ROBINSON, J. A., HIRSH, H. L., and DOWLING, H. F. (1949). *Ann. intern. Med.*, **30**, 398.

Caronamide undoubtedly reduces the wastage of penicillin through the kidneys. It is given in a dosage of 4 g. 4-hourly, but the large size of the tablets is apt to cause nausea. A simple emulsion containing 0.5 g. of the drug per teaspoon is available and is very useful for children. Roughly, caronamide will quadruple the effective levels of penicillin in the serum; it is more generally useful in clinical practice if given in association with oral penicillin than with intramuscular injections.

G. F. Walker.

Streptomycin in Non-tuberculous Infections. BUXTON, P. H., SIMON, R. D., and SELBIE, F. R. (1949). *Lancet*, **1**, 729.

Streptomycin was given in 67 cases, consisting of: (1) 44 cases of urinary infection, chiefly by *Pseudomonas aeruginosa* and *Bacterium coli*; (2) 14 cases of wound infection, mainly by *Staphylococcus pyogenes*; (3) 9 miscellaneous infections. The infecting organisms were proved to be sensitive *in vitro* to streptomycin, but insensitive to the sulphonamides and to penicillin. The dose used in urinary infections was 3 g. daily for 4 days, usually injected intramuscularly every 3 hours; potassium citrate was also given to keep the urine alkaline. If there was much pain at the site of injection, procaine was administered. For wound infections 4 g. was usually given daily for 2 days to obtain rapidly a high concentration in the tissues. Streptomycin was also injected subconjunctivally in cases of panophthalmitis, while cases of surface infection were treated by the local insufflation of a powder containing streptomycin 1 g., penicillin 45,000 units, and sulphathiazole 9 g. Of the 44 cases of urinary infection, including 10 cases with suprapubic drainage and 9 cases after prostatectomy, 14 were cured and 12 improved. The criteria of cure were sterile urine and absence of symptoms

of infection. In the cases in which suprapubic drainage was established there was a high proportion of failures due to reinfection. No advantage was gained by continuing treatment longer than 4 days, since the sensitivity of the organisms to streptomycin was then greatly reduced. Of the 14 cases of wound infection 10 were cured and 2 improved. Among the miscellaneous cases staphylococcal infections resistant to other forms of chemotherapy responded well.

It is suggested that if no improvement occurs within a week surgical treatment should be considered with a view to eliminating inaccessible foci of infection and providing adequate drainage. Reactions were encountered in less than a third of the cases. No auditory symptoms were observed, but vestibular disturbance was pronounced in 3 cases. Less serious reactions included pain at the site of injection, headache, and nausea in about 10 to 20% of the cases. *R. Wien.*

Neomycin, a New Antibiotic Active against Streptomycin-resistant Bacteria, including Tuberculosis Organisms. WAKSMAN, S. A., and LECHEVALIER, H. A. (1949). *Science*, **109**, 305.

A preliminary account is given of "neomycin," a new antibiotic active against mycobacteria. The organism producing neomycin, like that producing streptomycin, is a species of *Streptomyces* isolated from the soil and is related to *Streptomyces fradiae* isolated in 1915 by Waksman and Curtis. Neomycin is soluble in water and is bacteriostatic against numerous Gram-positive and Gram-negative bacteria, including *Mycobacterium phlei* and *M. tuberculosis*, the antibiotic spectrum of crude preparations being distinct from those of streptomycin and streptothricin. Neomycin is also highly bactericidal and has little or no toxicity to animals. Resistance to neomycin has not been found among the organisms sensitive to it, nor has more than a limited degree of resistance developed after prolonged incubation of such organisms in contact with the drug. The antibiotic has not yet been obtained in crystalline form, but preliminary chemical studies suggest a distinct difference from streptomycin and streptothricin.

Assay of Aureomycin in Body Fluids: Observations on Individuals Receiving Aureomycin. BRAINFERD, H. D., BRUYN, H. B., MEIKLEJOHN, G., and SCAPARONE, M. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 318.

Thirty individuals received aureomycin in doses varying from 0.16 to 2.5 g. orally, 50 to 100 mg. intravenously, and 50 to 200 mg. intramuscularly. After oral administration the drug was present in the blood after 1 hour and its concentration reached a maximum after 2 to 4 hours. The peak concentrations lay between 0.6 and 2.5 μg. per ml. of serum and measurable amounts persisted for over 6 hours. Intravenous doses of 50 mg. gave serum concentrations equal to or greater than those with 1 g. orally. Intramuscular doses of 50 to 200 mg., administered in a phosphate buffer with 2% procaine, led to a concentration of greater than 0.15 μg. per ml. in only one case. In urine the concentration of aureomycin varied between 5 and 50 μg. per ml. depending upon the route of administration and the time of collection.

Aureomycin in Typhus and Brucellosis. KNIGHT, V., RUIZ-SANCHEZ, F., RUIZ-SANCHEZ, A., and MCDERMOTT, W. (1949). *Amer. J. Med.*, **6**, 407.

Eleven patients with confirmed murine typhus were given 4 to 6 g. of aureomycin by mouth daily in divided doses. The clinical condition of the patients improved within 24 hours, the headache and gastro-intestinal symptoms disappearing overnight and fever in 24 to 72 hours. One patient who was afebrile, however, showed no signs of improvement.

Five young adults with brucellosis were treated by the same regimen. In all patients agglutinins for *Br. abortus* in titres varying from 1 in 80 to 1 in 640 were present in the serum. Febrile relapses occurred in 2 patients 14 and 42 days respectively after a course of aureomycin lasting for one week; this indicates that longer periods of therapy may be advisable. *R. Hare.*

BIOCHEMISTRY

Serum Level of Protein-bound Radioactive Iodine (I^{131}) in the Diagnosis of Hyperthyroidism. FREEDBERG, A. S., URELES, A., and HERTZ, S. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 679.

The levels of serum-protein-bound radioactive iodine were measured in 10 thyrotoxic patients and 10 with normal thyroid function. A fixed dose of 150 μc. carrier-free I^{131} was administered 3 hours after a light breakfast, and 10 ml. of blood was withdrawn 24 hours later. The protein-bound iodine was precipitated by cold 10% trichlorocetic acid. The total serum radioactivity was also measured. Results were expressed in terms of microcuries of radioactive iodine per ml. of serum. The "total serum radioactivity" values showed considerable overlap in the two groups of patients. The "protein-bound radioactive iodine" values, on the other hand, did not overlap; the values in hyperthyroid patients ranged from 38 to 146 $\times 10^{-5}$ μc. per ml. (average 68 $\times 10^{-5}$ μc. per ml.), and in patients with normal thyroid function from 3 to 28 $\times 10^{-5}$ μc. per ml. (average 13 $\times 10^{-5}$ μc. per ml.).

[This is a comparatively simple and promising test, but the absence of overlap will require confirmation in a larger series of cases.]

Studies in Cirrhosis of the Liver. I. Relationship between Plasma Volume, Plasma Protein Concentrations, and Total Circulating Proteins. HILLER, G. I., HUFFMAN, E. R., and LEVEY, S. (1949). *J. clin. Invest.*, **28**, 322.

Studies in 12 patients suffering from advanced Laënnec's cirrhosis of the liver showed that the serum-protein concentration was influenced by an associated increase in plasma volume determined by the Gibson and Evans dye method, so that a subnormal concentration did not necessarily indicate a subnormal quantity of total circulatory protein. Total blood volume was calculated from the plasma volume and venous haematocrit values. Liver function tests—thymol turbidity, cephalin-cholesterol flocculation, hippuric-acid excretion, and bromsulphalein excretion—gave abnormal results in all the patients studied. Of the 12 patients subjected to 80 tests, the total circulatory protein, calculated from blood volume and protein concentration, was found to be within theoretical normal limits in 69 instances. The total circulatory globulin value was above the theoretical normal in every instance tested. The degree of increase in plasma volume compared to the normal predicted volume was in some cases in the order of 5,500 ml. to 2,750 ml., but this showed no correlation with the presence or absence of ascites, anaemia, or oedema. Clinical improvement in several cases appeared to be related to the ability to maintain a level of total circulatory albumin above the predicted minimal value, even though the albumin concentration in the serum—often wrongly used as a guide to prognosis—was abnormally low.

The Effect of Desoxycorticosterone Acetate and Sodium Chloride, Thyroxin, and Testosterone Propionate in a Case of Panhypoadenopituitarism (Simmonds' Disease) with Special Reference to Kidney Function and Blood Pressure. [In English.] LUFT, R., and SJÖGREN, B. (1949). *Acta endocrinol., Kbh.*, **2**, 44.

A woman with severe Simmonds's disease was treated with desoxycorticosterone acetate and sodium chloride, testosterone propionate, thyroxine, and various combinations of these. Desoxycorticosterone alone caused only slight improvement in her general condition and a restoration of blood pressure to low normal levels. Testosterone caused no hirsuties or virilism, but there was a marked improvement in her general condition with an increase in weight and recovery of strength. Treatment by thyroxine resulted in a deterioration in her general condition, rapid loss of weight, and a serious hypoglycaemic attack. When thyroxine was later given in conjunction with testosterone propionate there was a rapid reappearance of axillary and pubic hair, the skin showed increased vascularity, and the basal metabolic rate rose to normal. The patient now became strong enough to get up. When desoxycorticosterone acetate and sodium

chloride were added there was a further increase in weight and she developed hypertension. Further slight increase in weight occurred when thyroxine was suspended.

Studies of renal function showed that some improvement in the previously low clearance values occurred when desoxycorticosterone acetate was given. The addition of thyroxine caused a decided increase in the clearance values. Hypertension did not occur with desoxycorticosterone when the renal clearances were very low, but it developed rapidly when the clearances were improved. The authors suggest that hypertension is not necessarily associated with an increase in blood volume but is dependent on renal circulation. *A. C. Crooke.*

Excretion of Chloride and Sodium after Surgical Operations. WILKINSON, A. W., BILLING, B. H., NAGY, G., and STEWART, C. P. (1949). *Lancet*, **1**, 640.

A study was made of the chloride and sodium excretion and nitrogen balance of 27 patients before and after operation, and of 4 healthy male volunteers. Of the healthy controls 2 received a diet providing 2,500 calories daily, with measured salt intake, while the caloric intake of the other 2 was restricted to 500 calories daily for 3 days, increasing thereafter as with a typical patient after gastrectomy. No anaesthesia or surgery was carried out on these controls, whose daily chloride excretion was found to vary with the intake of salt and to be unrelated to the protein intake.

A group of 5 patients undergoing partial gastrectomy was treated after operation by continuous intravenous infusion of saline or glucose solution and 2-hourly gastric aspiration for periods up to 4 days ; a similar group of 4 patients underwent abdominal and other operations after which intravenous infusion and gastric aspiration were not necessary. In both groups there was post-operative depression of urinary chloride excretion in spite of the intravenous administration in the former group of more than 10 g. of salt daily, the total loss of salt from gastric aspiration during the first 3 post-operative days being estimated at 5 to 15 g. A post-operative negative nitrogen balance, with striking increase in nitrogen excretion, coincided roughly with the period of diminished chloride excretion.

In the immediate post-operative period another group of 4 patients received intravenous casein hydrolysate in amounts calculated to yield an intake of approximately 11 g. of nitrogen daily, together with a higher chloride intake than in the pre-operative or later post-operative periods. Nevertheless, their chloride excretion fell after operation, returning to the pre-operative level after 5 to 8 days (at which time chloride intake was usually at its lowest owing to the transition from intravenous to oral feeding), while nitrogen excretion was increased coincidentally with the fall of chloride excretion. A mixture of whole milk, dried skimmed milk, and lactose, calculated to provide daily 90 g. protein and 1,700 calories, was given to a fourth group of 7 patients after operation, initially by double-lumen tube inserted at operation into the distal limb of the jejunum, and later by mouth. Their salt intake was never less than 4 g., and during intravenous infusion it was 10 g. or more daily. In 3 of these patients the daily urinary chloride excretion was less than 1 g., in 3 it was less than 2 g., and in one less than 3 g.

A further group of 7 patients were given a constant salt intake, and the sodium and chloride contents of their stools were measured as well as that of the urine. Excretion of sodium and chloride was reduced in 5 of these patients for the first 7 to 8 days after partial gastrectomy, the onset of the reduction being delayed until the second or third day in 2 cases. Although the retention of both ions continued for 8 days, chloride retention exceeded that of sodium from the third to the seventh days. In 2 patients who were observed for a long enough time this excess was found to be excreted later. The increase in nitrogen excretion and the coincident reduction in salt excretion was comparable in all groups and bore no relation to the amount of protein, salt, water, or calories taken, nor to the route by which they were given.

In view of these findings it is suggested that the retention of sodium and chloride is related to the inflammatory reaction to injury, that increased protein breakdown is another part of this reaction, and that the need for post-operative intravenous saline administration to combat a supposed state of salt depletion should be reviewed.

HAEMATOLOGY

A Case of Cyclical Agranulocytosis with Marked Improvement following Splenectomy. FULLERTON, H. W., and DUGUID, H. L. D. (1949). *Blood*, **4**, 269.

Cyclic Agranulocytosis. [In English.] OWREN, P. A. (1949). *Acta. med. scand.*, **134**, 87.

Two cases are described of cyclical agranulocytosis. In the first, a man aged 62, splenectomy was followed by a transient marked leucocytosis. The leucocyte count later fell to low levels but there was not complete agranulocytosis. The patient's general health improved considerably.

The second is of a man of 23 years. In many attacks polymorphs were absent from the blood for 3–5 days. The bone marrow was hypoplastic and there were increases in the numbers of plasma cells and reticulum cells. All medical treatment was unavailing; splenectomy produced only temporary benefit. The patient died after 5 years' observation.

Haemolytic Transfusion Reaction due to Anti-S. CUTBUSH, M., and MOLLISON, P. L. (1949). *Lancet*, **2**, 102.

The authors describe the discovery of the antibody anti-S in the serum of a woman (group A Rh negative) who had received repeated transfusions for an anaemia of obscure origin. By means of the indirect Coombs test it was shown that her serum contained an antibody active against 14 out of 17 Rh negative bloods chosen at random. When the serum was tested in parallel with a known anti-S serum the two sera reacted alike. As S negative blood was badly tolerated it was concluded that an additional antibody was present. Its presence could not, however, be demonstrated *in vitro*.

Chronic Lymphatic Leukemia. A Study of 100 Patients Treated with Radioactive Phosphorus. LAWRENCE, J. H., LOW-BEER, B. V. A., and CARPENDER, J. W. J. (1949). *J. Amer. med. Ass.*, **140**, 585.

The patients had been treated between 1936 and 1948; 58 of them received *x*-ray therapy in addition to P^{32}. Twenty-four patients were alive at the time of this study, 35% of them having survived for 6 years or more and 13% of them for 8 years or more. In many cases palliation was all that could be achieved. An absence of radiation sickness is a point in favour of P^{32} therapy.

The Coagulation Defect in Thrombocytopenic Purpura. QUICK, A. J., SHANBERGE, J. N., and STEFANINI, M. (1949). *J. Lab. clin. Med.*, **34**, 761.

The authors describe an attempt to correlate the prothrombin consumption in human plasma with quantitative estimates of clot retraction. In thrombocytopenic purpura delayed clot retraction and poor prothrombin consumption were associated with a subnormal platelet count. The prothrombin consumption test is considered to provide a measure of platelet activity.

The Clinical Association of Macrocytic Anemia with Intestinal Stricture and Anastomosis. CAMERON, D. G., WATSON, G. M., and WITTS, L. J. (1949). *Blood*, **4**, 793.

The case history is described of a woman who developed a macrocytic megaloblastic anaemia after an anastomosis had been performed to short circuit a stricture due to regional ileitis. Intramuscular liver therapy had no effect, but oral liver extract and proteolysed liver were effective in treatment. It is concluded that the essential causative factor was stasis of bowel contents in the by-passed loop.

MORBID ANATOMY AND HISTOLOGY

A Tuberculin-neutralizing Factor in the Serum of Patients with Sarcoidosis. WELLS, A. Q., and WYLIE, J. A. H. (1949). *Lancet*, **1**, 439.

The authors found that blood serum from certain patients suffering from clinically-diagnosed sarcoidosis contains a factor capable of neutralizing "old tuberculin," so that the latter no longer evokes a skin reaction in tuberculin-sensitive subjects, or modifying the action of the tuberculin so that only a diminished reaction results. The test is of no use for diagnosis of sarcoidosis, however, because the neutralization phenomenon occasionally occurs with normal serum or with the serum of patients suffering from other diseases; moreover, neutralization may not take place if the sarcoidosis is relatively inactive, as, for example, during a spontaneous remission of the disease or after effective radiotherapy.

A similar tuberculin-neutralizing factor has been demonstrated in the serum of some patients with leishmaniasis, and it would thus seem to be a feature of diseases which have as a pathological characteristic the mobilization of epithelioid cells. It may, therefore, be present in berylliosis.

Electrophoretic fractionation of serum from cases of sarcoidosis shows that the tuberculin-neutralizing factor is all in the γ-globulin. It is heat-labile and is unaffected by soya-bean antitrypsin, thus differing from fibrinolysin. The point is of interest because fibrinolysin can neutralize the ability of other antigens, such as pollen, to cause a skin reaction in a sensitive subject. The tuberculin-neutralizing factor in sarcoidosis, on the other hand, does not affect these antigens. The presence of the factor in the γ-globulin is noteworthy, for in many cases of sarcoidosis a proportional increase in serum globulin has been observed. It would be of great interest to know whether the tuberculin-neutralizing factor is present in other conditions associated with a rise in serum globulin. *John Forbes.*

Lipocalcinogranulomatosis—a New Lipoidosis. (Die Lipocalcinogranulomatose—eine neue Lipoidose.) ANDREAS, E. (1949). *Med. Klinik*, **44**, 913.

A case is described in which symmetrical firm tumours were situated just above the olecranon processes of each elbow. The patient, a child of 10½, had had the lump in the left arm for a year, and in the right for 6 months. The tumours were attached to the deep structures, not to the skin, and there was some limitation of movement at the elbow joints.

Radiographs showed irregular, calcified masses in the region of the triceps tendons. Histologically, the "tumours" consisted of areas of necrosis in the triceps muscle and tendon, with a deposit of doubly-refracting crystals and substances taking up fat stains. These areas were surrounded by epithelial cells and foreign-body giant cells.

The concentration of lipid in the affected tendon was found to be 8 times the normal value; the author therefore claims that the condition be regarded as a special form of lipidosis and compares the clinical and biochemical findings with those in other types. *H. K. Goadby.*

A Case of Transplacental Metastasis of Malignant Melanoma from Mother to Foetus. HOLLAND, E. (1949). *J. Obstet. Gynaec. Brit. Emp.*, **56**, 529.

A woman of 25 was delivered of a full-term infant by Caesarean section eleven months after a recurrent malignant melanoma had been excised from the thigh. The placenta and an ovary contained secondary growth on histological examination.

The woman died 3 months later; there is no report of a necropsy. The infant died at the age of 10½ months of widely disseminated neoplasm, and a post-mortem examination was carried out. The histology of the neoplasms in the various specimens was similar throughout. The malignant cells would appear to have reached the placenta by embolism along the maternal blood vessels and to have crossed the placental barrier into the foetal circulation.

Six similar cases found in the literature are reviewed, and it is pointed out that this case is the first reported in which thorough histological examination of all the relevant tissues has yielded convincing evidence of the process by which the neoplasm reaches the foetus.

Neuritis and Polyneuritis after Typhoid–Paratyphoid Vaccination. A Further Contribution to the Subject of Allergy and the Nervous System. BANNWARTH, A. (1948). *Arch. Psychiat. Nervenkr.*, **118**, 531.

This is a description of 8 cases in which peripheral nerves were affected by typhoid–paratyphoid vaccination. In 3 patients there was acute neuritis of the brachial plexus; in 2 cases bilateral polyneuritis of the lumbo-sacral plexus; and the remaining 3 patients had a typical polyneuritis of the Guillain–Barré type. The author believes that these cases should be regarded as allergic manifestations similar to polyneuritic conditions seen in serum sickness or the rheumatic diseases.

Sarcoidosis. A Clinicopathologic Review of Three Hundred Cases, Including Twenty-two Autopsies. RICKER, W., and CLARK, M. (1949). *Amer. J. clin. Path.*, **19**, 725.

The authors analyse 300 cases of sarcoidosis taken from the records of the Army Institute of Pathology, Washington. The report is based on cases diagnosed by surgical biopsy and followed up by means of a questionary, in addition to details of findings at necropsy in 22 of the cases. Histological criteria of diagnosis were used in selecting the material for study. The organs showing the highest incidence of involvement at necropsy were the lungs, liver, lymph nodes, and spleen.

Observations on the Pathology of Hydrocephalus. RUSSELL, D. S. (1949). *Spec. Rep. Ser. med. Res. Counc., Lond.*, No. 265, 1.

A study of the morbid anatomy and histology of internal hydrocephalus, with illustrative cases. It is concluded that obstruction at some point of the cerebrospinal pathway is the operative factor in all, and that the term "idiopathic" may be regarded as obsolete. The different sections deal with maldevelopments, gliosis of the aqueduct, inflammations, and dural sinus-thrombosis as factors in the causation of hydrocephalus. A final chapter gives some account of the anatomical effects of hydrocephalus, both general and local.

Parafollicular Cell Adenoma of the Thyroid Gland. BAKAY, L. (1948). *Arch. Path.*, **45**, 447.

A goitre of a new histological type, to which the name "parafollicular-cell adenoma" has been given, was found in 3 women, aged 38, 29, and 23 years respectively, at the Neurosurgical Clinic, Stockholm. By comparing the microscopical structure of the lesion in the 3 cases, the development of the adenoma could be followed. First, small follicles are formed containing a small amount of colloid and surrounded by numerous capillaries. Later, the cells and their nuclei become larger and have the appearance of parafollicular cells (but without the increased activity of the latter, as was shown by the benign clinical symptoms), the nucleus being pushed to the side of the cell opposite to the capillaries and near to the colloid. As the transformation of thyroid tissue into adenoma becomes complete, the cells enlarge and the nuclei become shrivelled, resembling the "oncocytes" described by Hamperl, which the author regards as degenerate varieties of parafollicular cells. Finally, the follicles are dissolved, the colloid disappears, and the cells become grouped round the vessels in rosette form with the pyknotic nucleus withdrawn to the end of the cell away from the lumen of the capillary. The author concludes that this type of adenoma is histologically identical with the Hürthle-cell tumour of Wilensky and Kaufmann, but differs from the large-cell goitre of Langhans. *Wilfred E. Hunt.*

Peripheral Neuritis in Periarteritis Nodosa : A Clinicopathologic Study. LOVSHIN, L. L., and KERNOHAN, J. W. (1949). *Proc. Mayo Clin.*, **24**, 48.

Of 29 cases of periarteritis nodosa studied there were clinical signs of peripheral neuritis in 15. This neuritis tended to appear early, to be widespread and severe, and to affect the lower limbs and the motor rather than the sensory neurones. A clinical regression occurred in 4 cases. Comparison of this group of 15 cases with the remainder did not reveal any significant difference in the incidence of hypertension, fever, leucocytosis, or eosinophilia. Necropsy in 25 of the cases revealed involvement of the nutrient arteries in 19 and in all of the cases with clinical signs of peripheral neuritis. The nerve degeneration was Wallerian in nature and there was no sign of an inflammatory neuritis. *R. N. Johnston.*

The Pathological Physiology of the Bone Marrow in Pernicious Anaemia. The Problem of the Megaloblast. FRUHLING, L., ROGER, S., and SPEHLER, H. (1949). *Sang*, **20**, 164.

Sternal puncture in 40 cases of pernicious anaemia, in some repeated, indicated that the administration of liver extract or of folic acid caused the new formation of normoblasts from their precursors, while pre-existing megaloblasts degenerate *in situ*.

The authors conclude that: (1) megaloblasts cannot be transformed into normoblasts ; (2) megaloblasts are cells suffering from simultaneous disorders of development and maturation ; and (3) " intermediate " megaloblasts are megaloblasts suffering from a minor degree of disordered development ; they are not transitional stages between megaloblast and normoblast.

Primary Amyloidosis, with Report of Six Cases. DAHLIN, D. C. (1949). *Amer. J. Path.*, **25**, 105.

Cases of primary amyloidosis have no history of tuberculosis or chronic suppuration. The organs most commonly affected are the heart, lungs, striped muscle, and other non-abdominal structures, the abdominal viscera are involved less commonly, and there are occasional amyloid tumours. The staining reactions of the amyloid substance are atypical.

Of 57 cases reported in the literature the necropsy findings were given in 54, amyloid change being present in the various organs as follows: heart 46, gastro-intestinal tract 33, tongue 26, lungs, 24, striped muscle 19, liver and kidneys 21, spleen 20. Bones, joints, brain, peripheral nerves, and other organs were also involved on occasion. In some cases the intravenous Congo red test had been negative.

The clinical histories, necropsy findings, and histological appearances in 6 new cases are reported. The author comments on the remarkably diffuse involvement of mesodermal structures and points out that the characteristic distribution patterns of primary and secondary amyloidosis overlap. In the primary form the cardiac lesions are clinically the most important and macroglossia less important than has previously been maintained. When the amyloid substance does not take up intravenous Congo red, then biopsy is necessary before a diagnosis can be made. No clue to the pathogenesis of primary amyloidosis has been found. *R. R. Wilson.*

Significant Factors in the Causation of Biliary Cirrhosis. SCHMITZ, R. C., and SINAIKO, R. P. (1949). *Amer. J. digest. Dis.*, **16**, 121.

This is a detailed analysis of 93 cases of biliary cirrhosis discovered during 3,922 post-mortem examinations over a period of 18 years. The main causes were as follows: carcinoma of the pancreas, 28.9% ; stones in the common bile duct, 21.5% ; carcinoma of the bile duct, 17.4% ; and carcinoma of the gall-bladder, 12.9%.

[For detailed statistical data concerning the incidence of the various signs, symptoms, and pathological changes encountered the paper should be consulted in the original.] *R. Schneider.*

Pancreatic Atrophy in Infants with Fatty Liver. [In English.] HARTZ, P. H. (1949). *Docum. Neer. indon. morb. trop.*, **1**, 41.

Recent work on kwashiorkor and fatty liver disease has demonstrated the presence of pancreatic atrophy, which has been attributed to dietary deficiency and is considered to precede the hepatic changes. In the present investigation the pancreas was studied in 14 infants in whom advanced fatty infiltration of the liver was found at necropsy. In only one of these had the fatty liver syndrome been observed during life. In all of them advanced acinar atrophy was found, with a preponderance of intercalated ducts. The islets were normal. Advanced atrophy was not found in control cases, but slight and moderate changes occurred in some. The absence of the syndrome of fatty liver disease in cases in which advanced pancreatic changes were seen suggests that the initial lesion of the syndrome is in the pancreas and that the infiltration of the liver takes place because of pancreatic dysfunction. *J. L. Markson.*

Primary Plasma-cell Tumors of the Upper Air Passages and Oral Cavity. STOUT, A. P., and KENNEY, F. R. (1949). *Cancer*, **2**, 261.

A useful review is given of 104 recorded cases (including 9 personally studied) of primary plasma-cell tumours of the mucous membranes of the head and neck. These are four times as frequent in men as in women, and three-quarters of them develop in the fifth, sixth, and seventh decades. They occur rather more frequently in the nasal cavity, sinuses, or nasopharynx than in the oral cavity and oropharynx. In two-thirds of cases there are no metastases; in the remaining one-third, metastasis occurs to the cervical lymph nodes or infrequently to bones or other sites. When metastases occur in bones, it is difficult or impossible to decide whether the case is indeed one of primary plasmocytoma in the oral or nasal mucosa or one of myelomatosis. *R. A. Willis.*

Synovial Sarcoma and Relatively Benign Synovioma. MUIRHEAD, E. E., KREISSL, L. J., and GORDON, C. E. (1949). *Tex. J. Med.*, **45**, 202.

The authors report 8 cases of synovial sarcoma, 4 cases of relatively benign synovioma, and 5 sarcomata of "questionable" synovial origin. *W. E. Tucker.*

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MENINGEAL TUMOURS: A REVIEW

BY

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The tumours to be considered here are those arising primarily in the meninges, or gaining access to them as secondary growths. It will be necessary to consider not only the dura and leptomeninges that invest the brain and spinal cord, but also their extensions into the brain as the tela choroidea and stroma of the choroid plexuses, and the sheaths of the perforating blood-vessels that form the outer boundary of the Virchow–Robin spaces. In the vertebral canal the separation of the dura from the bone by the epidural adipose and areolar tissue, and the resolution of the arachnoid and pia into two distinct membranes, introduce additional factors that are of importance, especially in the direct spread of tumours and the dissemination of their cells by the cerebrospinal fluid.

Although little has been heard of it lately, the old controversy as to whether the meninges are of mesodermal or of ectodermal origin cannot be regarded as satisfactorily resolved. In practice, however, it is undisputed that the primary meningeal tumours (meningiomas) display many features in common with mesodermal tumours in other parts of the body; the question of embryological origin is thus purely academic.

PRIMARY TUMOURS

The most important group to be considered includes the different varieties of meningioma. Before dealing with these it will be convenient to review a number of rarer tumours, composed of tissues or cells ordinarily foreign to the meninges, which are to be regarded as derivatives of embryonal remnants. These in general mostly occupy the leptomeninges in the mid-sagittal plane over the surface of the brain, though they may be laterally situated. In the vertebral canal they are often associated with congenital malformations, especially spina bifida.

Epidermoid and Dermoid Cysts

These are not sharply separable as distinct groups. The epidermoid, in which the squamous epithelial lining of the cyst is devoid of skin appendages, is far commoner than the classical dermoid. The best known form is the pearly tumour of Cruveihier, which arises in the leptomeninges as a smooth, often lobulated mass with a characteristic sheen derived from the concentrically laminated exfoliated keratin, mixed with cholesterin, which fills the interior and imparts so brittle a character to the mass. These tumours, which may develop to a considerable size, are usually found at the base of the brain either in the mid-line or in the cerebello-pontine angle;

occasionally in a lateral ventricle or over the corpus callosum. The lining of the cyst is a simple squamous epithelium. Smaller cysts lined with a similar epithelium have been identified in the pineal body and spinal cord. In the latter situation they are usually situated in the pia, but they may be embedded in the substance of the cord (Falconer and Hooper, 1941 ; Black and German, 1950). Epidermoid cysts and cystic tumours situated in the region of the sella turcica are usually not pearly and are often partly solid growths derived from remnants of Rathke's pouch. This conception of their origin is strongly supported by the frequency with which small nests of squamous cells can be identified under the microscope in and about the pituitary gland, its stalk and the adjacent leptomeninges. The content of these cysts is usually more or less fluid, turbid and brownish, with a suspension of abundant cholesterin crystals. The lining of the cyst may, as in the pearly tumours, be of a simple squamous character. More often, however, it is elaborated into papillary and trabecular formations which include cells of basal type as well as Malpighian cells, supported by connective tissue which is apt to undergo mucinous degeneration. Calcification and even bone-formation are frequent secondary changes in the solid parts of the tumour. Those forms in which the basal cells are conspicuous often receive the name of " adamantinoma." The propriety of using this term is, however, questionable since there is no sure evidence that these cells have any relationship to the ameloblasts of the enamel organ ; the resemblance is but superficial. The *dermoid cysts* are not clearly separable from the epidermoid. In fact Montgomery and Finlayson (1934) described a tumour, incorporated in the dura of the middle and posterior fossae, which was in part epidermoid only but in part dermoid. Occasionally cysts arise in the vermis which earn the title of dermoid from the presence within them of hairs, but are otherwise epidermoid. The fully developed dermoid cyst (Fig. 1) is rare ; that illustrated, which showed all the usual skin appendages, occupied the basal part of the frontal lobes.

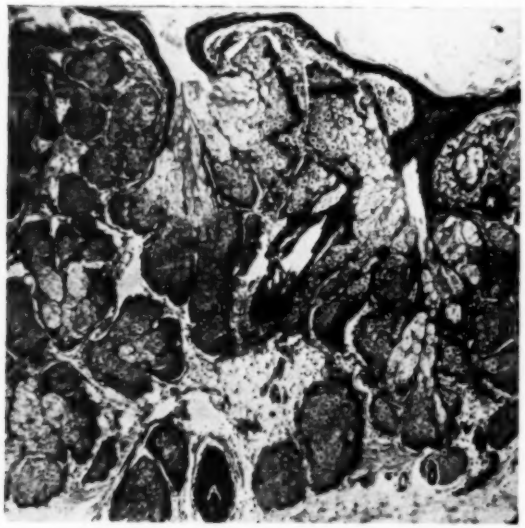

FIG. 1.—Dermoid cyst from base of brain showing sebaceous glands and hair-follicles. H. and E. × 65.

Other teratoid tumours and typical teratomata may be encountered within the skull and vertebral canal but more frequently involve the tissues of the central nervous system than its integuments. In the spinal cord, however, they may abut on the meninges. A series of 15 such tumours arising in childhood has recently been described by Ingraham and Bailey (1946) ; eight of these were intracranial and seven intraspinal. Of the latter, five were associated with spina bifida.

Lipoma

Islands of adipose tissue, representing another variety of these embryonic remnants, occasionally manifest themselves as lipomata of the leptomeninges. As a rule they remain small, and are merely pathological curiosities disclosed during the routine examination of the brain. Bailey and Bucy (1931) found records of 60 reported cases. They usually lie over the corpus callosum or the tuber cinereum ; occasionally they are embedded superficially in the corpora quadrigemina, but have not been known to excite symptoms with the possible exception of Taubner's case (quoted by Bailey and Bucy, *loc. cit.*).

In the spinal cord larger lipomata may arise at the site of a spina bifida, and are sometimes intimately blended with the cord in addition to the meninges. A more diffuse involvement of the cord by lipomatous tissue, apart from spina bifida, may be accompanied by neurological symptoms, as in the well-documented case of Baker and Adams (1938), in which lipomata were found in the choroid plexus of the left lateral ventricle, the left cerebello-pontine angle, and throughout almost the whole length of the spinal cord.

Melanoma

A well-defined though rare condition, recognized first by Virchow (1859) and named " melanosis " of the meninges, is clearly a naevoid form of neoplasia in which the leptomeninges over the base of the brain are especially apt to be affected. There is diffuse pigmentation from the infiltrating cells, which may penetrate deeply into the nervous tissue along the sheaths of perforating vessels. Hydrocephalus is a recognized sequel (Russell, 1949). Extensive cutaneous pigmented naevi may be associated with the cerebral abnormality. These cases have been reviewed by Schnitker and Ayer (1938).

Circumscribed melanotic tumours of the meninges and adjacent brain substance have also been reported by several observers, and doubtless certain of these are acceptable as arising primarily in this situation. But doubt must be entertained concerning this point in examples where a complete necropsy has not been performed, and there are many reports in which fuller particulars are desirable. The histological appearances may be those of a malignant tumour ; even when histologically benign the liability to recurrence after operation seems to be considerable (Christensen, 1941). It is desirable to distinguish between this group and the occasional examples of meningioma, to be described later, in which melanin pigment may be produced by the tumour cells.

Chordoma

Remnants of notochordal tissue may linger in the region of the basisphenoid and basiocciput, and give rise either to ecchordoses or chordomas, which are usually disclosed in the adjacent meninges. The ecchordosis, a chance finding at necropsy, is a small jelly-like mass of greyish-white tissue which generally lies over the basilar artery, and is attached by a delicate stalk to the dura over the clivus. The notochordal cells of which it is composed are usually in advanced stages of degeneration ; the mass is essentially inert and incapable of further development.

The chordoma, on the other hand, is an invasive tumour capable of penetrating the meninges and exerting pressure within the cranium, or upon the nerve-roots

in the sacro-coccygeal region. Since these tumours are related primarily to the bone rather than the meninges they will not be discussed further.

Meningioma

This group is one of the most important of the intracranial tumours, forming from 12 to 16% of the total in large published series from various neurosurgical centres. The name now so generally adopted was originally introduced by Cushing in 1922. Its significance is clearly generic, and it comfortably evades the controversy that inevitably ensues when attempts are made to devise a suitable name with cytological implications for a group of tumours with the most diverse microscopical appearances. The old name "dural endothelioma" held pride of place until it came to be recognized, following the work of M. B. Schmidt (1902), that the source of origin of such tumours lay in the nests of arachnoid cells included in the dura as arachnoid villi (Fig. 2). But neither "arachnoid fibroblastoma" (Mallory, 1920) nor "meningeal fibroblastoma" (Penfield, 1927) can be regarded as satisfactory equivalents for a tumour which often appears quite devoid of fibroblasts.

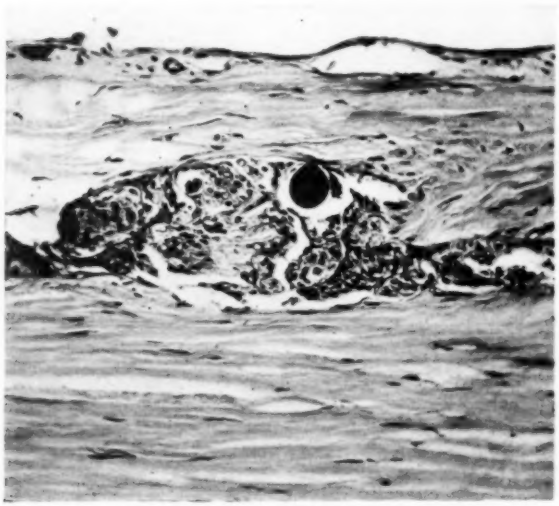

FIG. 2.—Arachnoid villus projecting into dural vein remote from a main sinus. The dark circular mass is a calcospherite. H. and E. × 160.

The diversity of cell type that goes to form the meningiomas is best understood in recollecting that the arachnoid cells form the macrophages of the cerebrospinal pathway in the meninges, are vitally stainable with trypan blue and are thus a component of the reticuloendothelial system. Their polyblastic character is reflected then in the cytological and architectural variations in the neoplasms to which they give rise, and this has found expression in a considerable number of suggested classifications. The best known of these are by Bailey and Bucy (1931), Globus (1937), Cushing and Eisenhardt (1938), and del Rio Hortega (1941). I attempted to tabulate these in terms of their synonymous types, but the existence of transitional forms that are recognized and named by some workers but not by all, and the consequent lack of a precise equivalence in the definition of the accepted types by these authors, made the task impracticable. Again variations in experience have led to the inclusion of sundry rare types in one classification or another, but not in all: for example, chondroblastic meningiomas (Cushing

and Eisenhardt), and melanotic (Bailey and Bucy). It is perhaps better to pass by these discrepancies and difficulties, with the recognition that they exist, than to elaborate their details. For Cushing and Eisenhardt (p. 54) frankly state: "We regard most of the subdivisions, i.e., variants, under a given type as fundamentally unimportant. Indeed, we find, somewhat to our surprise, that there is essentially little difference even between certain of the main types, either from the standpoint of histo-functional differentiation or life history of the tumour." Further, as will be shown, any rigid attempt to relate histological varieties to one or other of the three meninges as the tissue of origin is to invite contradictory experience.

Cushing's opinion is certainly endorsed by the results of tissue-culture in various histological types of meningioma (Bland and Russell, 1938). We found a considerable range of variation in the patterns of growth and in the morphology of the cells, ranging from sheets of flat, polygonal cells of "epithelioid" appearance to spiky growths of narrow spindle cells. But these variations appeared in different cultures of the same tumour, and could not be correlated closely with the morphology of the cells of the parent tumour.

It is proposed, therefore, to adopt the simpler scheme of Courville (1945), who suggested five main types: (i) syncytial, (ii) transitional or mixed, (iii) fibrous, (iv) angioblastic, and (v) sarcomatous. These are tolerably well recognizable as equivalents in the other main classifications. After summarizing these some attention will be devoted to the rarer varieties that perhaps hardly merit recognition as "types."

(i) Syncytial Type (Meningotheliomatous meningioma of Bailey and Bucy; leptomeningioma of Globus; meningothelial meningioma, Type I, of Cushing and Eisenhardt; exotelioma difusa of Rio Hortega).—This form, as the name suggests, is composed of sheets of polygonal cells subdivided irregularly by vessels and collagen fibres; fibroglial fibrils and reticulin are absent (Fig. 3). The cell-boundaries are often poorly defined. This type was designated "endotheliomatous" by Bland and Russell (1938) and we found that it accounted for 56.6% of our series.

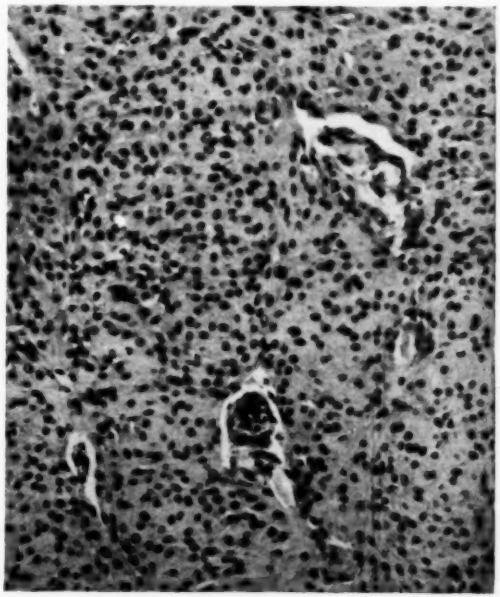

FIG. 3.—Meningioma: syncytial type. H. and E. × 165.

(ii) Transitional Type (Psammomatous type of Bailey and Bucy would be included here; also the "primitive meningioma" of Globus; meningothelial Type II of Cushing and

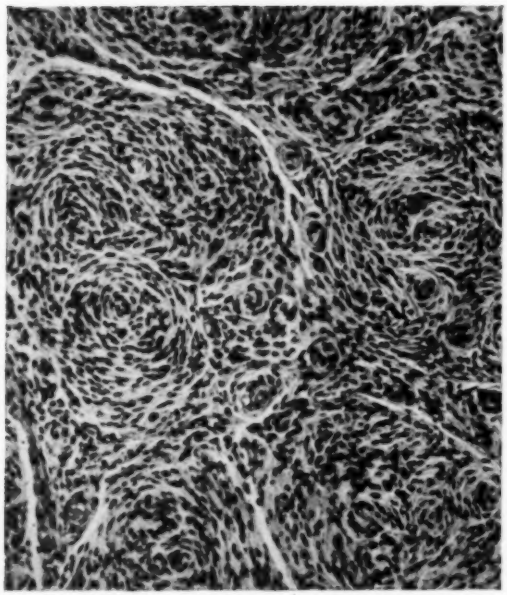

FIG. 4.—Meningioma: transitional type. H. and E. × 133.

Eisenhardt ; exotelioma nodular and lobulado of Rio Hortega). —It is generally agreed that many examples of the syncytial type show a greater or less tendency to the formation of whorls, in which groups of the cells are wrapped round one another recalling the appearance of an onion in transverse section (Fig. 4). The intervening cells between the whorls tend to be spindle-shaped and to contain fibroglial fibrils. This tendency is recognized by Cushing and Eisenhardt in their description of four variants of Meningothelial Type I, and in their separation of a Meningothelial Type II in which the outstanding feature is the predominance of whorls and their further conversion into psammoma-bodies. There is obviously no sharp distinction here, but a smoothly graded series in which the progressive development of whorls and psammoma-bodies, separated by collagen and spindle cells, comes to dominate the histological picture. A convincing interpretation of this ultimate structural arrangement has been advanced by Orville Bailey (1940), who likens the polygonal cells to the functional arachnoid cells of the villi and Pacchionian bodies, where they form the cap cells ; whilst the spindle cells, containing fibroglial fibrils, are interpreted as stroma cells such as the covering arach-

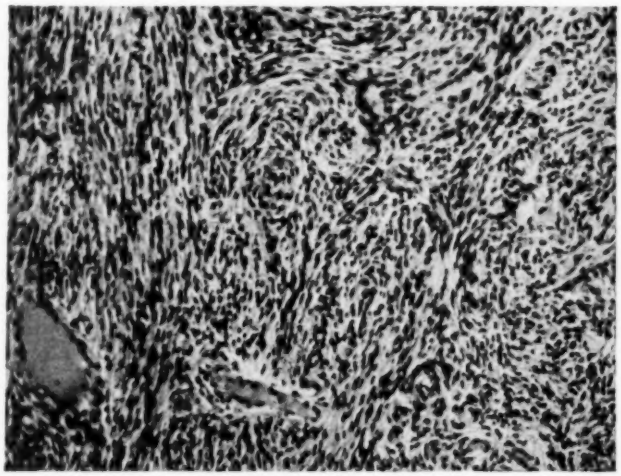

FIG. 5.—Meningioma: fibroblastic type. H. and E. × 123.

noid cells require for their support, and might be expected to evoke. Bailey found no evidence that the fibroblastic cells were derived by differentiation from the arachnoid type of cell. Moreover the rate of growth and prognosis do not appear to differ substantially with the development of fibroblastic cells within these tumours. The clinical features of these two types, syncytial and transitional, which together accounted for 65% of Cushing and Eisenhardt's series, thus reflect in no way their histological diversity.

(iii) Fibroblastic (Bailey and Bucy; Cushing and Eisenhardt; dural fibroblastoma of Globus; exotelioma laminar of Rio Hortega).—The fibrous meningioma of Courville may also be included, but his description of the additional features of whorls and psammoma bodies does not allow of a clean separation from (ii). The tumour described by Bailey and Bucy is regarded by Cushing and Eisenhardt as being rather of a fibrosarcomatous nature; the reason for this is not clear since it seems to have behaved like a benign tumour. In general, however, this type is rich in stout collagen fibres, between which the spindle cells lie. These cells form interweaving bundles, and occasionally loose whorls such as are seen in the acoustic nerve tumours (Fig. 5). Fibroglial fibrils are conspicuous (Fig. 6). Though some are attached to the dura, excellent examples may arise from the pia of the spinal cord, and the intraventricular meningiomas are generally of this group. The term "dural" fibroblastoma (Globus) is therefore inappropriate.

FIG. 6.—Meningioma: fibroblastic type showing fibroglial fibrils in cells. P.T.A.H. × 440.

(iv) Angioblastic (Bailey and Bucy; Cushing and Eisenhardt; Globus classifies this as a sub-type both of pial meningioma and of leptomeningioma; Rio Hortega separates it from the meningioma group altogether).—This is a distinctive type of cellular tumour in which numerous small and larger blood-spaces of capillary structure are separated by plump polygonal cells which are often foamy and may show mitosis (Fig. 7). Fine reticulin fibrils form a dense network throughout the tumour (Fig. 8). Some of these tumours are identical in appearance with the haemangioblastoma of the cerebellum (Lindau's tumour), and in this connexion it is of interest that Cushing noted their predilection for the tentorium and the peritorcular region. But undoubtedly a good many are of pial origin and we have encountered a classical

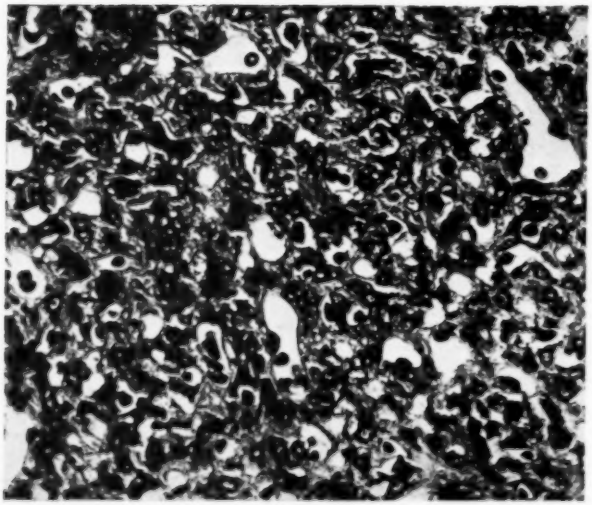

FIG. 7.—Meningioma: angioblastic type. P.T.A.H. × 380.

example arising in the pia of the spinal cord and in the leptomeninges of the cerebrum. It is therefore pertinent to inquire into the propriety of including the cerebellar tumours in this group; a question upon which there is no agreement. These cerebellar tumours are cystic or solid growths arising in the mid-line or laterally; occasionally the tonsil is the site of origin. Others, however, may spring from the dorsal aspect of the medulla oblongata, in the region of the area postrema. In the cystic examples the wall of the cyst is composed of smooth neuroglial tissue which is occupied at some point by a reddish-brown nodule of the tumour proper, the "mural nodule," which may be very small in proportion to the whole. The cyst contains clear straw-coloured fluid, rich in protein, which is regarded as a transudate from the capillaries of the tumour nodule. Microscopically the tumour is composed of a mesh of blood-spaces, of capillary structure but of varying calibre, supported by a stroma of plump polygonal cells. The cytoplasm of these cells appears vacuolated in paraffin sections, and is characteristically laden with doubly refractive Sudanophil material in frozen section. Reticulin stains show a wealth of fine fibrils

FIG. 8.—As in Fig. 7 to show reticulin. Laidlaw × 380.

supporting these cells and forming the basement-membranes of the blood-spaces.

The obvious similarity between these tumours and the angioblastic meningiomas has provided the theme of several studies (Wolf and Cowen, 1936 ; Bailey and Ford, 1942 ; Corradini and Browder, 1948) in which the attempt has been made to decide whether or not they were clearly separable. In general the influence of Bailey, Cushing, and Eisenhardt (1928), who originally favoured their separation, seems to have prevailed. The chief grounds for this view lie in the apparent dural origin of most of the supratentorial tumours and their non-cystic character, whereas the cerebellar examples appear to be embedded in the neural tissues. No single criterion, however, appears absolute in actual experience. The supratentorial examples may arise independently of the dura, as already noted. Further they may be cystic, as in the case reported by Barnard and Walshe (1931), though this is clearly exceptional judging by the lack of similar cases in the literature. The cerebellar haemangioblastoma may be solid and, although it is regarded as being intracerebellar and usually devoid of any attachment to the leptomeninges (Corradini and Browder), there are reasons for doubting this. We have already noted (Bland and Russell, 1938) that " many of these cerebellar tumours are contiguous with, and in some cases involve, the pia." I have recently reviewed a series of 33 cases of this group obtained both from biopsy and necropsy in this Institute. There is evidence of direct continuity between the tumour and the pia in 21 of these ; in the remainder (biopsies) the material is insufficient for the analysis. It is proposed to substantiate these observations in a separate communication. Here it must suffice to state that the relationship between these cerebellar haemangioblastomas and the pia appears to be of so intimate a character in such a large proportion of examples that their pial origin cannot reasonably be denied. The reader's attention is directed to Fig. 106 on p. 148 of Cushing and Bailey's monograph (1928) illustrating the presence of tumour tissue over the surface of the cerebellar cortex in an example of cerebellar haemangioblastoma, and to the article by Bailey and Ford (1942) in which (case 2) a cerebellar tumour of this type is described as arising from the leptomeninges, and is interpreted by the authors as transitional between the two groups of tumour under discussion.

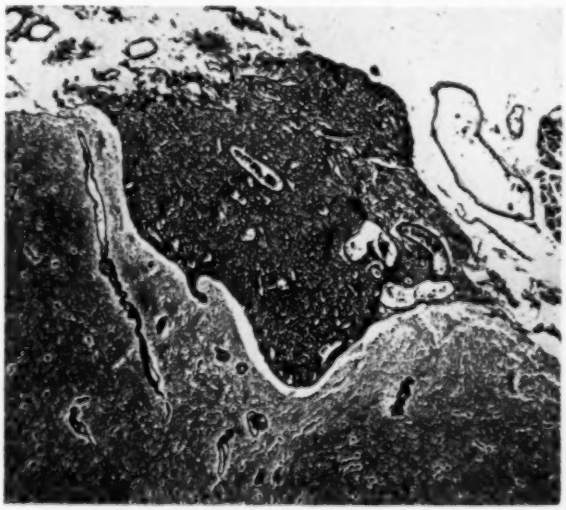

FIG. 9.—Capillary haemangioblastoma in pia over spinal cord in a case showing Lindau's syndrome. Laidlaw × 27.5.

It must also be noted that the capillary haemangioblastoma can occur in the spinal

cord and, when deeply embedded in the cord tissue, is liable to be complicated by syringomyelia. But again there is continuity between the tumour and the pia and examples are occasionally encountered in which the pial origin of the tumour is obvious (Fig. 9). This illustration is taken from a case that displayed the features of Lindau's syndrome (infratentorial capillary haemangioblastoma with multiple pial tumours in the spinal cord, von Hippel's disease, multiple cysts of the pancreas and solid and cystic tumours of benign Grawitz type in the kidneys).

Lindau (1931) listed angioma of the retina (von Hippel's disease), cystic pancreas, cysts of the kidneys and " hypernephromata of the kidneys or the suprarenal glands " as the ingredients of the syndrome that goes by his name. He particularly mentioned the cystic pancreas as characteristic. This curious assortment of pathological conditions constitutes an inherited disease, but accompanies a small proportion only of these infratentorial haemangioblastomas, and has not so far been recorded in association with the supratentorial angioblastic meningiomas. This alone suggests that the infratentorial tumours possess an individuality that demands their consideration as a separate group.

FIG. 10.—Meningioma of spinal theca containing much lamellar bone. H. and E. × 11.

Histological Variations.—Before proceeding to the malignant, or sarcomatous, forms of meningiomas it is proposed to deal briefly with certain histological variations which, if rare, are nevertheless important in gaining a general view of these neoplasms and their potentialities. These are concerned with the presence of bone, cartilage, melanin, and doubly refractive Sudanophil fatty substances.

(*a*) *Bone.*—Meningiomas of the first three types described above occasionally contain bone, which may be coarse-fibred or lamellar or both. Sometimes the bone is laid down in relation to calcified psammoma bodies, but usually it is independent of these. This should not be confused with the well-known hyperostosis of the adjacent cranium which will be considered separately. Indeed, in our experience, it is the spinal examples that are most liable to be osteoblastic (Fig. 10). The anatomical separation here of the dura from the bone exemplifies well the distinction between these two features.

(*b*) *Cartilage.*—The presence of cartilage in meningiomas must be exceedingly rare. Bailey and Bucy do not mention it, and in Cushing's series there is only one

such case, which proved to be a chondrosarcoma. Cushing and Eisenhardt mention seven others from the literature, one of which (Wolf and Echlin, 1936) also was malignant. Recently Forsythe, Baker, Dockerty, and Camp (1947) in reporting a large osteochondroma, apparently arising in the dura over the cerebral convexity and falx in the same style as a meningioma, state also that approximately 38 cases of primary solitary intracranial cartilaginous tumours had been reported to date. It is doubtful how many of these should be interpreted as chondroblastic meningiomas in the sense described by Cushing and Eisenhardt. Some may indeed arise from cartilaginous rests, as suggested by Wolf and Echlin, but they also pointed out that such rests had not been identified in the dura or pia-arachnoid. I have, however, once encountered a cartilaginous rest in the falx of a kitten; the possibility is thereby strengthened.

(*c*) *Melanin.*—This pigment is normally present in the pial cells over the ventral surface of the medulla oblongata and upper cervical cord; it is less frequent elsewhere. Melanin therefore appears in occasional examples of meningioma, particularly of the posterior fossa and spinal cord. Surprisingly this phenomenon is not mentioned by Cushing and Eisenhardt. Bailey and Bucy describe one example, in a girl of 9, who had multiple small pigmented tumours in the cerebral leptomeninges and in the floor of the fourth ventricle, without evidence of a primary source elsewhere in the body. Microscopically the tumours were composed of polyhedral cells containing melanin and scantier spindle cells in bundles. Abundant reticulin fibres divided the tumour cells into alveoli. Mitoses were noted as "common throughout." The account of this case raises considerable doubt concerning its real nature. The peculiarities of malignant melanomas in general certainly invite caution in their interpretation. Occasionally, however, a meningioma is encountered with the characteristic appearances of the transitional or fibroblastic type but differing in the conspicuous, though often patchy, development of melanin granules in the cytoplasm of the tumour cells.

(*d*) *Fatty Substances.*—Meningiomas of types (i) and (iv) quite often contain so much intracellular Sudanophil lipoid that they are opaque and yellowish macroscopically, and foamy in stained paraffin sections (Fig. 11). The appearances suggest a storage process rather

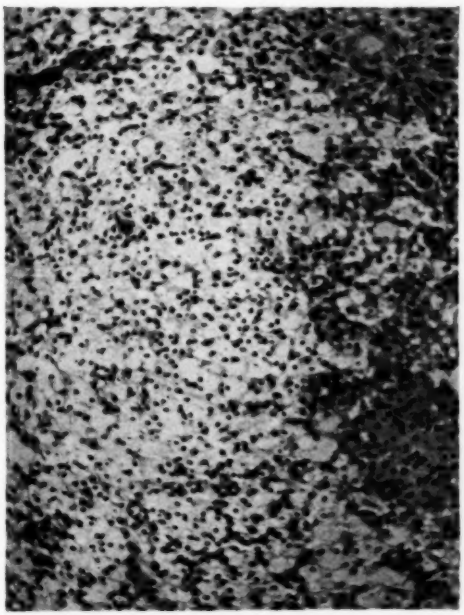

FIG. 11.—Meningioma of syncytial type with conspicuous xanthomatous areas. H. and E. × 109.

than degeneration since the nuclei are well preserved and the lipoid is doubly refractive.

(v) Sarcomatous Meningiomas.—The forms so far considered have been benign. When incompletely removed they usually recur, but it is seldom that they display a greatly increased rate of growth, histological evidence of malignancy, or diffuse invasion of the adjacent brain. But when these occur there may be a striking alteration in the cytological appearances and architecture of the tumour, as in Cushing and Eisenhardt's case, Dorothy May Russell, who underwent 17 operations for removal of recurrent growth and finally, at necropsy, was found to have pulmonary metastases. Starting as a spindle-celled tumour this example ended with a papillary epithelial structure which was reflected in the metastases. I have twice observed a similar papillary appearance in undoubted meningiomas (Fig. 12). Malignancy was suggested in one of these by invasion of the brain; in the other, in a girl of 16, a fatal recurrence of the growth followed operative removal. Though Cushing and Eisenhardt attributed sarcomas to the fibroblastic type of meningioma, and thus wrote of fibrosarcomas, it seems that potential malignancy is not the exclusive attribute of any one type. The angiomatous meningioma in particular carries a relatively poor prognosis, being of rapid growth and apt to recur after radical treatment, and prone to invade the brain at late stages. Histologically this tumour often contains a considerable number of mitotic figures.

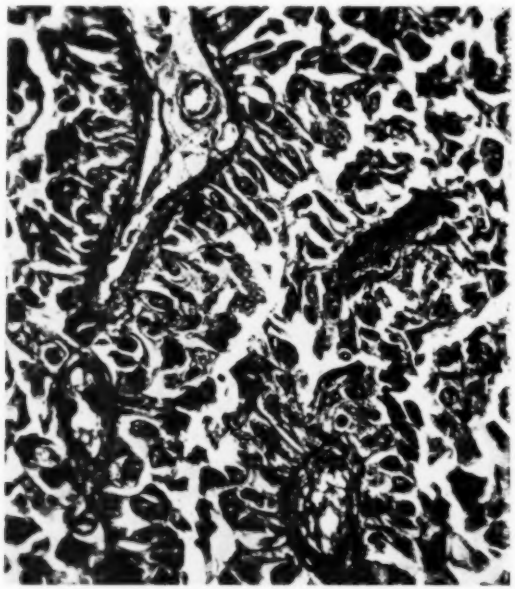

FIG. 12.—Meningioma with papillary structure. Silver carbonate × 350.

Swingle (1949), however, has reported a case in which, like Cushing's, the tumour was of fibroblastic character. Eight years after radical treatment a recurrence was partly removed and the patient, a male aged 39, died five years later with pulmonary metastases. An example, recently examined in this Institute, is of interest in showing a profound alteration in the cytology and general character of a frontal parasagittal meningioma at successive operations. The patient, a man aged 24, was admitted first to the London Hospital in 1948 under the care of Mr. D. W. C. Northfield. The tumour removed then was a well-defined growth of typical and benign appearance, with numerous whorls separated by short spindle cells (Fig. 13). As shown in the photomicrograph a narrow fringe of cerebral tissue, adherent in places

to the surface, is indented by bosses of tumour tissue, but not obviously invaded. The recurrence, removed a year later, is a spindle-celled fibrosarcoma with great variation in the size of the cells and the chromatin content of their nuclei. Mitoses are numerous (Fig. 14). It invades the brain diffusely, with the segregration of islands of neural tissue within its advancing borders (Fig. 15). This case is of importance not only in showing this change of cell type in the acquisition of malignancy, but also in supporting Orville Bailey's view that the fibroblastic type of meningeal cell is not a product of differentiation.

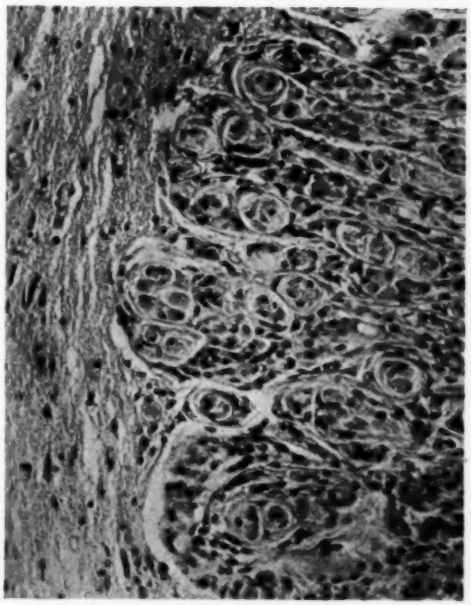

FIG. 13.—Meningioma removed at first operation showing benign, whorled type of growth, and its relationship to adjacent cerebral tissue. H. and E.× 175.

Occasionally no such transition from a benign to a malignant form of meningioma is observed : the tumour is a sarcoma when first seen. A group of this kind has been described by Globus and others (1944). Particularly when such tumours are disclosed in children and young subjects it seems likely that they have been sarcomatous from the start. Histologically they may be highly cellular fibrosarcomas, or of a primitive mesenchymal character as exemplified in a recent report of a fibro-myxosarcoma of the skull and meninges in a child of 3 (Baker, Dockerty, and Kennedy, 1950).

Metastasis.—In Cushing and Eisenhardt's malignant example, mentioned above, a sarcomatous change in the primary tumour was followed by pulmonary metastasis, disclosed at necropsy. There are few other records of blood-borne metastasis, and the meningiomas appear to share with other intracranial tumours a notable reluctance to spread in this way. Abbott and Love (1943) reported a malignant haemangioblastic meningioma of the frontal region in a man of 32 ; this, after two operations for resection of the growth combined with radium therapy, metastasized as nodules of firm grey tissue to all lobes of both lungs. The total length of the history was three years and nine months. Other metastasizing meningiomas have been reported by Jurow (1941, one case), Russell and Sachs (1942, three cases), Hamblet (1944, one case), and Swingle (*loc. cit.*).* The cases of Jurow and Hamblet are remarkable for the typical appearances of the secondary deposits, which displayed well-developed whorls of arachnoid-cell type. The authenticity of Russell and Sachs' three cases has been questioned by some writers, in particular their case 2 in which 13 years intervened between an operation for the removal of the meningioma (which did not recur locally) and the appearance of metastases

* Recently Christensen, Kiaer, and Winblad (1949) have reported two further examples.

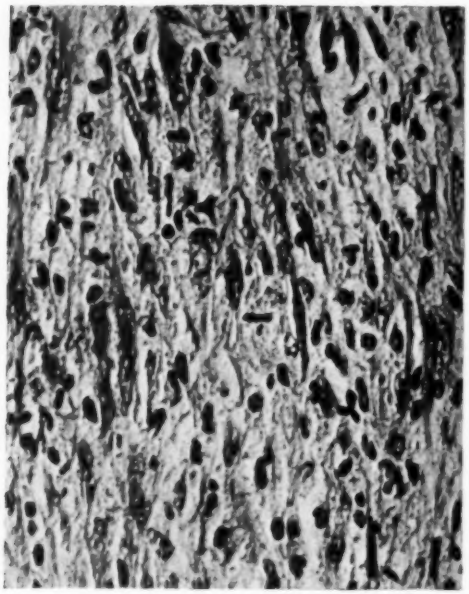

FIG. 14.—Meningeal fibrosarcoma from second operation (see Fig. 13). Mitosis at centre of field. H. and E. × 370.

elsewhere. Their case 3 is certainly difficult to interpret, but their case 1 appears to be indisputable. Six of the reported cases are therefore acceptable.

Sites of Origin and Methods of Local Spread.—With few exceptions there is no close association between the histological varieties of the meningiomas and their topography. While most of them are firmly attached to the dura over the brain or spinal cord, a few are found to embed themselves in the nervous tissues, and to be attached to the pia or leptomeninges only; an important group is related to the ventricles of the brain. The origin of many of the dural tumours is traced to the presence within that membrane of nests of arachnoid cells, forming the villi and Pacchionian bodies. Hence the meningiomas frequently arise in relation to the main dural venous sinuses, especially the anterior half of the superior longitudinal sinus. Their origin at more remote sites over the cerebral convexities may be traced to the development, increasing with age, of similar arachnoid nests as pointed out by Schmidt (1902). Alternatively, as Orville Bailey has argued (1940) a meningioma arising primarily in the leptomeninges may gain a secondary dural attachment upon breaching the arachnoid membrane.

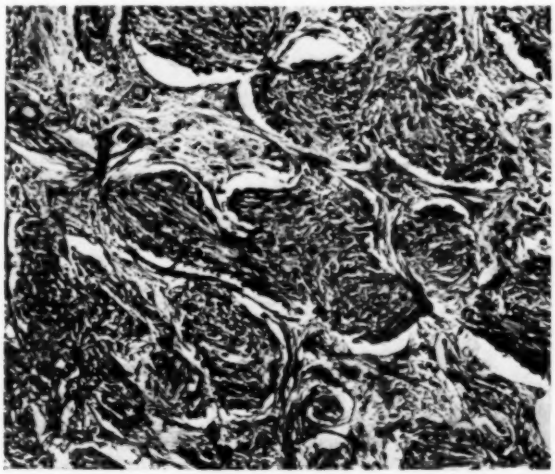

FIG. 15.—Same specimen as in Fig. 14; margin of growth showing inclusion of islands of nervous tissue (lighter areas) within spindle-celled growth. Masson's trichrome × 130.

Clinically the anatomical situation of these tumours is the predominant consideration, and they are usually classified accordingly in practice rather than upon the more academic aspects of their cytology. Such an arrangement is found in Cushing and Eisenhardt's important monograph, to which the reader is referred for fuller details. Here it must suffice to point out that the meningiomas frequent the anterior part of the cranium more than the posterior. The parasagittal examples may penetrate the falx to become embedded in both frontal lobes ; they have been carefully studied by Olivecrona (1934). The lumen of the sinus is often invaded, and even occluded, by the growth but without metastasis taking place. The region of the Sylvian fissure as it traverses the ventro-lateral border of the cerebrum, the olfactory groove (Fig. 16), the tuberculum sellae, the lesser wing of the sphenoid, the cavernous sinus, and the sheath of the optic nerve are additional sites. The last mentioned is relatively rare, but a meningioma may arise here, especially in childhood, as a dumb-bell mass constricted by the optic foramen. Craig and Gogela (1950) have reported two cases of bilateral and one of unilateral foraminal tumours with slowly progressive blindness as the sole symptom.

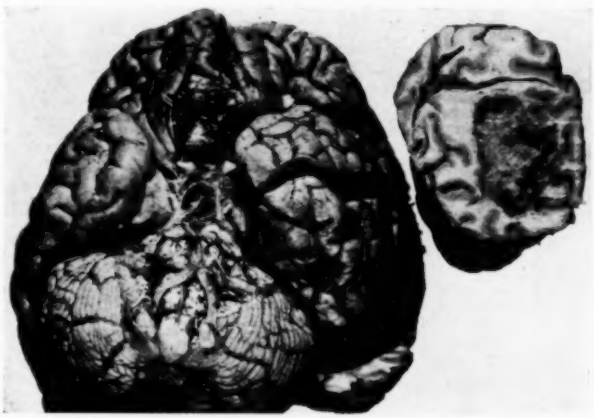

FIG. 16.—Combination of meningioma of olfactory groove and spongioblastoma multiforme of left occipital pole.

In relation to the posterior part of the brain, meningiomas may arise over the convexities, the main dural sinuses including the torcular, from the posterior aspect of the petrous bone or in relation to the foramen magnum.

As already mentioned meningiomas of fibroblastic type may occur in the ventricles. Here they arise in the stroma of the choroid plexus, or in the velum interpositum. It is remarkable that, like the choroid plexus papilloma, they seem to have a preference for the left lateral ventricle which is gradually distended by a firm globular mass of growth, to the surface of which the remnant of the plexus is attached. It has been argued as to whether the tumour arises in the stroma of the plexus at the confluence of the occipital and temporal horns, or whether it extends laterally into the ventricle from the tela choroidea, pushing the plexus before it. Support is gained for the former view by the observation in this Institute (P.M. 92.1945) of a small example measuring $2.5 \times 2 \times 1.5$ cm. at this point of the plexus (Fig. 17). The tumour was symptomless. The intraventricular meningiomas have been reviewed by Abbott and Courville (1942), who found reports of 50 cases in the literature. As they point out, the tumour may occasionally take the form of a sausage-like thickening of the whole length of the plexus. A remarkable

FIG. 17.—Small meningioma arising in choroid plexus.

example of this, affecting both plexuses of the lateral ventricles in a symmetrical fashion, occurred in this Institute many years ago (P.M. 36.1924) in association with von Recklinghausen's neurofibromatosis. The case is briefly mentioned by Cushing and Eisenhardt (p. 114). In distinction to the usual fibroblastic type, the tumours in this case were highly psammomatous.

Corresponding tumours of the fourth ventricle seem to be far rarer and more debatable; they are fully discussed by Cushing and Eisenhardt.

The meningiomas of the vertebral canal are the commonest tumour of this region according to some, but in other neurosurgical centres the nerve-root tumours slightly preponderate. As in the cranium, they are usually attached to the inner aspect of the dura. The arachnoid villi, from which they arise, are formed where the arachnoid membrane is brought into apposition with the dura at the emergence of the nerve roots. Occasionally, however, the tumour arises in the pia and becomes deeply embedded in the substance of the cord. The commonest levels at which the meningiomas occur are the thoracic segments, according to Elsberg (1941), who had a unique experience with these and other spinal-cord tumours. He records that 80% of his 73 cases were in women.

Hyperostosis.—In addition to penetration through the dura and occupation of adjacent venous sinuses, the convexity meningiomas show a notable tendency to invade the overlying bone, and may even penetrate the pericranium, muscles, and cavities of air-sinuses. But such invasion is no indication of malignancy, in terms of cytological change or remote metastasis. Involvement of the bone usually leads to a conspicuous thickening, or hyperostosis, of the part affected. The mechanism of this bony change is still warmly debated. Bailey (1940), Courville (1947), and Freedman and Forster (1948) have discussed the rival theories. From these and other papers it is clear that there is no agreement as to whether or not the tumour cells are directly responsible for the deposition of new bone. Obviously, as already noted, the meningiomas are capable of producing bone within their substance. But tumours displaying this propensity seem seldom to be associated with a hyperostosis of their cranial attachment. On the other hand there is a good deal to suggest that the meningioma, like certain carcinomatous metastases, exerts an osteoplastic effect in which the tumour cells themselves cannot be directly responsible for bone-production. Thus in some examples of convexity meningioma the inner table of the adjacent skull shows a nipple-like protrusion towards the centre of the attached growth, without histological evidence of invasion of the bone by growth. The medullary spaces of the region are occupied by fibrous tissue containing scanty spindle cells. Another possible factor, responsible perhaps for the "sun-ray" pattern of some hyperostoses in *x*-ray photographs, is the stimulation of periosteal

bone-formation which, following Ribbert (quoted by Courville and Crockett, 1948), is due to the stretching of vessels entering Volkmann's canals when a mechanical strain is exerted upon the periosteum or dura. Such a pattern is characteristic both of certain primary bone-tumours and of some secondary deposits, such as those derived from a neuroblastoma of the suprarenal medulla, that cause an elevation of the periosteum.

The incidence of hyperostosis in meningiomas varies widely in different reported series. Figures ranging from 25% to 4.5% are quoted by Courville, who considers that the smaller figure is the more acceptable. Undoubtedly the tumours related to the vault of the skull are those most productive of hyperostoses, especially when related to the intersections of the mid-sagittal and coronal suture-lines (Cushing and Eisenhardt). From this it has been considered that diastasis of the sutures from trauma may be an important aetiological factor. Nevertheless hyperostosis may also accompany tumours at the base of the skull, particularly those overlying the lesser wing of the sphenoid, and occasionally those over the tuberculum sellae.

Multiplicity and Associations with Other Types of Primary Growth.—Multiple tumours of the meninges and of other regional tissues are usually the manifestation of the central type of von Recklinghausen's neurofibromatosis. But occasionally two or more meningiomas may be found in a case that displays no evidence of this disease. We have observed seven examples of this kind at necropsy. Usually a large tumour, producing clinical symptoms, is associated with an unsuspected, small, and remotely situated tumour. Mufson and Davidoff (1944) have reviewed the literature and report two additional cases, in one of which 10 meningiomas were removed from the right cerebral convexity at four operations. At necropsy several more were found, including a flattened mass in the right side of the tentorium. When multiplicity is an accompaniment of von Recklinghausen's neurofibromatosis the meningiomas are associated with bilateral acoustic-nerve tumours, and often with other primary growths of the cranial and spinal nerve-roots ; gliomas may also be found.

Apart from neurofibromatosis there are also instances in which an intracranial meningioma has been associated with a glioma in some other area of the cerebrum. Such an example has been reported by Alexander (1948), who finds only four similar cases in the previous literature and concludes, from the rarity of the association, that it is probably coincidental. A fifth has been reported by Kirschbaum (1945): the combination of an intrasellar meningioma with multiple cerebral glioblastomas. The latter, from his description, probably represented intraventricular metastases and not independent growths as he postulated. Examination of the spinal cord, which might have settled this point, was not carried out. We have observed three examples in which a meningioma was associated with a glioma ; the latter was invariably a spongioblastoma multiforme (Fig. 16).

Reticuloses

Allowing that the cells of the leptomeninges form part of the reticulo-endothelial system, it is in no way surprising that these should on occasion participate in the cellular proliferations that affect this system in other parts of the body. But such proliferations involve principally the lymphadenoid tissues, and hence it is mainly in the manifestations known as lymphosarcoma and reticulo-

sarcoma that the meninges, and their extensions along the perforating vessels of the central nervous system, may be involved.

Hodgkin's disease is, in its histological aspects, a granulomatous form of cellular proliferation. But there are many who prefer to regard it as a neoplasm and there is certainly a form, often referred to as Hodgkin's sarcoma, which macroscopically and microscopically lends some support to this interpretation. Involvement of the nervous system in this form of Hodgkin's disease is discussed by Sparling, Adams, and Parker (1947) and briefly by Jackson and Parker (1947). In the first of these two contributions the authors describe three cases in which tumour masses were identified within the brain substance ; in one there seems little doubt that this was the primary site to be involved. The relationship between such tumours and the reticulosarcomas, and also the proliferations within the central nervous system that have been described by Russell, Marshall, and Smith (1948) as "microgliomatosis," is at present impossible to decide. Since the microglial cells are of mesodermal origin and can therefore undoubtedly be derived, amongst other sources, from the perivascular sheaths, their proliferation within the brain is primarily related to the blood-vessels with the production of perivascular cellular foci, and tumours arise by the confluence of these foci. Jackson and Parker regard the reticulum cell as identical with the histiocyte, and therefore the microglial cell. Since the careful application of silver techniques reveals important differences in the affinities for silver on the part of these cells (Robb-Smith, 1938) there are grounds for disagreeing with this assumption. The reticulum cell should preferably be regarded as a more primitive type, but capable of differentiating into a variety of forms including the histiocyte. Further observations will doubtless clarify the relationships between microgliomatosis and the reticulosarcomas of the brain (also discussed by Sparling and others, 1947, and by Kinney and Adams, 1943).

The reticuloses are perhaps more important in their relation to the spinal cord. Hodgkin's lymphogranuloma, and the form termed by Robb-Smith (1938) "lymphoreticular medullary reticulosis," sometimes give rise to masses in the epidural fat, causing compression of the cord. Hodgkin's lymphogranuloma affecting the vertebrae, or paravertebral soft tissues, may also invade the spinal roots and dura but seldom penetrates the arachnoid membrane. Nevertheless instances are occasionally encountered in which even the substance of the spinal cord is invaded by direct extension.

In *generalized lymphosarcomatosis* and in *myeloid leukaemia* extensive infiltration of the epidural tissues by tumour-like masses may lead to paraplegia, either from compression of the spinal cord or ischaemia resulting from local circulatory disturbances. Four such examples, complicating leukaemia, were reported by Critchley and Greenfield (1930), who reviewed the literature and discussed the pathology at length.

SECONDARY TUMOURS

Of Dura Mater

Secondary deposits in the dura are usually the outcome of a direct spread from foci of metastatic growth in the adjacent bone. In childhood these are most commonly derived from a primary neuroblastoma of the suprarenal medulla and as a rule the periosteum of the bones tends to be involved. Hence the dura is often elevated by bosses of soft grey tissue in which new bone is usually laid down.

In the adult, carcinomatosis of the bones may include either the vault or the base of the skull, or both. The dura is then often diffusely infiltrated and thickened by plaques of the growth which replace the normal glistening under-surface of the meninx. In some instances extensive permeation of the veins and capillaries of the dura leads to varying degrees of pachymeningitis interna haemorrhagica and even subdural haematoma (Russell and Cairns, 1934). At the base a similar permeation may lead to profound disturbance of pituitary function, and even acute pituitary cachexia from necrosis of the anterior lobe when the nutrient vessels of the gland are extensively permeated.

Of Leptomeninges

Secondary tumours here may be discrete or diffuse, the latter giving the appearances of a chronic meningitis. As a rule they are dependent upon the presence of a neoplasm within the central nervous system which, by the involvement either of the surface of the brain or cord, or of the ventricular ependyma, is enabled to metastasize by way of the cerebrospinal fluid. Such neoplasms may be either primary, or blood-borne metastases from a remote site.

Primary Tumours of the Central Nervous System.—These tumours, especially the gliomas, are now well recognized as capable of metastasizing in this way. The medulloblastoma of the cerebellum, being a highly malignant tumour, is a notable offender, and its situation in relation to the fourth ventricle and basal cisterns is peculiarly favourable to such spread. The metastases are usually discrete though poorly defined ; they may be massive and confluent. In the spinal cord they tend to involve the posterior surface rather than the anterior, and the cauda equina is a favoured site. All types of glioma, even the most differentiated, are, however, capable of behaving in a similar way. Tarlov and Davidoff (1946) have reported three cases in which spinal deposits complicated an ependymoma. The oligodendroglioma, long regarded as a relatively benign type, may spread as vigorously in the leptomeninges as any medulloblastoma and prove as rapidly fatal ; on the other hand the cells may provoke a chronic reactive meningitis and the clinical picture of internal hydrocephalus, which may be prolonged for as much as seven years (Beck and Russell, 1942) or even 14 years (Blumenfeld and Gardner, 1945). The naked-eye appearances, in such protracted cases, may be very difficult to distinguish from chronic fibrous meningitis. The primary tumour, which will probably be discovered in relation to the third or a lateral ventricle, may be so small that it has excited no localizing symptoms, and hence may be missed unless the brain is carefully examined after fixation.

Such possibilities need to be recalled before any example of gliomatosis of the meninges is classified as a primary condition ; indeed it is questionable whether such occurs. In any doubtful case the spinal cord should be examined, because an intramedullary glioma at any level is capable of spreading through the meninges, even to the surface of the cerebrum. The literature relating to this interesting phenomenon has been reviewed by O'Connell (1946), with the addition of three personal observations. An intramedullary oligodendroglioma of the cervical enlargement which spread in this way, causing internal hydrocephalus as the principal clinical disturbance, has been described by Russell (1949).

Secondary Tumours within the Brain.—These tumours may involve the leptomeninges by direct extension at the surface. But whereas the gliomata, particularly spongioblastoma multiforme, may gain attachment to the opposing dura this is less often seen in carcinomatous metastases.

Of greater interest is the diffuse carcinomatosis, or meningitis carcinomatosa, that may arise when a secondary deposit within the brain breaks through the ependyma of the adjacent ventricular wall. The meningeal spread may then constitute the main cause of clinical symptoms. Evidence of chronic meningitis, supported by the presence of excess of protein and mononuclear cells in the cerebrospinal fluid, may be combined with symptoms of cranial nerve-root involvement resulting from their infiltration with tumour cells. Recently a remarkable example was personally observed in which the cerebral metastasis, situated in the floor of the left lateral ventricle, was quite small (Fig. 18) and symptomless. But metastases in each foramen of Luschka gave rise to a clinical picture suggesting bilateral acoustic nerve tumours. The primary growth was in a bronchus.

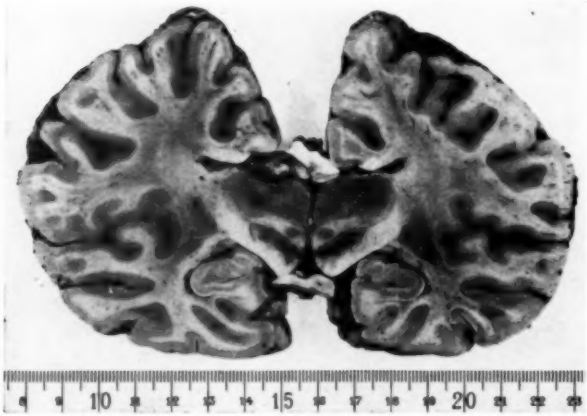

FIG. 18.—Coronal section of brain showing small metastasis from primary growth of bronchus in floor of left lateral ventricle. Metastasis by C.S.F. gave rise to bilateral tumours of cerebellopontine angle.

A similar diffuse spread of growth through the leptomeninges, especially at the base of the brain, may complicate primary carcinomas of the upper part of the nasopharynx or of the middle ear. Special mention should also be made of the malignant gliomas of the retina which are apt to extend along the optic nerve to the chiasma and thence throughout the leptomeninges of the brain and spinal cord. The growth in such cases may be luxuriant, forming thick sheets of soft tissue that obscure the blood-vessels and nerve-roots at the base of the brain.

But in most instances of leptomeningeal metastasis, whether this be related to a primary tumour of the brain or to secondary carcinoma, the meningeal deposits are not conspicuous to the naked eye. On close scrutiny they are detected as thin, greyish-white plaques that obscure the surfaces of the subjacent structures. They are most readily detected where they overlie sulci. Microscopically there is a variable degree of low-grade inflammatory response in relation to these deposits, and in places it will be found that the tumour cells penetrate the brain along the perivascular sheaths of the perforating vessels.

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References

Abbott, K. H., and Courville, C. B. (1942). *Bull. Los Angeles neurol. Soc.*, **7**, 12.
—— and Love, J. G. (1943). *Ann. Surg.*, **118**, 343.
Alexander, W. S. (1948). *J. Neuropath. exp. Neurol.*, **7**, 81.
Bailey O. T. (1940). *Arch. Path.*, **30**, 42.
—— and Ford, R. (1942). *Amer. J. Path.*, **18**, 1.
Bailey, P., and Bucy, P. C. (1931). *Amer. J. Cancer*, **15**, 15.
—— Cushing, H., and Eisenhardt, L. (1928). *Arch. Path.*, **6**, 953.
Baker, A. B., and Adams, J. M. (1938). *Amer. J. Cancer*, **34**, 214.
Baker, G. S., Dockerty, M. B., and Kennedy, R. L. J. (1950). *Proc. Mayo Clin.*, **25**, 129.
Barnard, W. G., and Walshe, F. M. R. (1931). *J. Path. Bact.*, **34**, 385.
Beck, D. J. K., and Russell, D. S. (1942). *Brain*, **65**, 352.
Black, S. P. W., and German, W. J. (1950). *J. Neurosurg.*, **7**, 49.
Bland, J. O. W., and Russell, D. S. (1938). *J. Path. Bact.*, **47**, 291.
Blumenfeld, C. M., and Gardner, W. J. (1945). *Arch. Neurol. Psychiat., Chicago*, **54**, 274.
Christensen, E. (1941). *Acta chir. scand.*, **85**, 90.
—— Kiaer, W., and Winblad, S. (1949). *Brit. J. Cancer*, **3**, 485.
Corradini, E. W., and Browder, J. (1948). *J. Neuropath. exp. Neurol.*, **7**, 299.
Courville, C. B. (1945). *Pathology of the Central Nervous System*. 2nd ed., Pacific Press, California.
—— (1947). *Bull. Los Angeles neurol. Soc.*, **12**, 6.
—— and Crockett, H. G. (1948). Ibid., **13**, 86.
Craig, W. McK., and Gogela, L. J. (1950). *J. Neurosurg.*, **7**, 44.
Critchley, M., and Greenfield, J. G. (1930). *Brain*, **53**, 11.
Cushing, H., and Bailey, P. (1928). *Tumors Arising from the Blood-vessels of the Brain*. Springfield, Illinois.
—— and Eisenhardt, L. (1938). *Meningiomas*. Springfield, Illinois.
Elsberg, C. A. (1941). *Surgical Diseases of the Spinal Cord, Membranes and Nerve Roots*. New York.
Falconer, M. A., and Hooper, R. S. (1941). *Brit. J. Surg.*, **28**, 538.
Forsythe, R. W., Baker, G. S., Dockerty, M. B., and Camp, J. D. (1947). *Proc. Mayo Clin.*, **22**, 350.
Freedman, H., and Forster, F. M. (1948). *J. Neuropath. exp. Neurol.*, **7**, 69.
Globus, J. H. (1937). " Tumors of the Nervous System ": *Proc. Ass. Res. Nerv. Ment. Dis.*, 1935, **16**, 210.
—— Levin, S., and Sheps, J. G. (1944). *J. Neuropath. exp. Neurol.*, **3**, 311.
Hamblet, J. B. (1944). *Arch. Path.*, **37**, 216.
Ingraham, F. D., and Bailey, O. T. (1946). *J. Neurosurg.*, **3**, 511.
Jackson, H., and Parker, F. (1947). *Hodgkin's Disease and Allied Disorders*. New York.
Jurow, H. N. (1941). *Arch. Path.*, **32**, 222.
Kinney, T. D., and Adams, R. D. (1943). *Arch. Neurol. Psychiat., Chicago*, **50**, 552.
Kirschbaum, W. R. (1945). *J. Neuropath. exp. Neurol.*, **4**, 370.
Lindau, A. (1931). *Proc. Roy. Soc. Med.*, **24**, 1.
Mallory, F. B. (1920). *J. med. Res.*, **41**, 349.
Montgomery, G. L., and Finlayson, D. I. C. (1934). *Brain*, **57**, 177.
Mufson, J. A., and Davidoff, L. M. (1944). *J. Neurosurg.*, **1**, 45.
O'Connell, J. E. A. (1946). *J. Neurol. Neurosurg. Psychiat.*, **9**, 55.
Olivecrona, H. (1934). *Die parasagittalen Meningeome*. Leipzig.
Penfield, W. (1927). *Surg. Gynec. Obstet.*, **45**, 178.
Rio Hortega, P. del (1941). *Arch. argent. Neurol.*, **24**, 7.
Robb-Smith, A. H. T. (1938). *J. Path. Bact.*, **47**, 457.
Russell, D. S. (1949). " Observations on the Pathology of Hydrocephalus." *Spec. Rep. Ser. med. Res. Coun., London*, No. 265.
—— and Cairns, H. (1934). *Brain*, **57**, 32.
—— Marshall, A. H. E., and Smith, F. B. (1948). Ibid., **71**, 1.
Russell, W. O., and Sachs, E. (1942). *Arch. Path.*, **34**, 240.
Schmidt, M. B. (1902). *Virchows Arch.*, **170**, 429.
Schnitker, M. T., and Ayer, D. (1938). *J. nerv. ment. Dis.*, **87**, 45.
Sparling, H. J., Adams, R. D., and Parker, F. (1947). *Medicine, Baltimore*, **26**, 285.
Swingle, A. J. (1949). *Arch. Neurol. Psychiat., Chicago*, **61**, 65.
Tarlov, I. M., and Davidoff, L. M. (1946). *J. Neuropath. exp. Neurol.*, **5**, 213.
Virchow, R. (1859). *Virchows Arch.*, **16**, 180.
Wolf, A., and Cowen, D. (1936). *Bull. neurol. Inst. N. Y.*, **5**, 485.
—— and Echlin, F. (1936). Ibid. **5**, 515.

J. clin. Path. (1950), 3, 212.

TWO CASES OF HUMAN INFESTATION BY LARVAE OF LINGUATULA SERRATA

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Human infestation by *Linguatula serrata*, one of the family of linguatulids or so-called tongue-worms, is uncommon; the recorded cases appear without exception to have been diagnosed at necropsy, where the parasites were unimportant incidental findings. In an exhaustive survey of the literature we have been unable to find any published record* of a case of infestation by this parasite occurring in the British Isles, and the presence of its larvae in the liver in two patients in Birmingham is therefore of some interest, particularly as the specimen in one case was obtained surgically. Neither patient had ever been out of the country.

The Linguatulids

The linguatulids are obligate endoparasites belonging to the phylum Arthropoda, within which their precise position seems controversial; they are usually assigned to the order Acarina in the class Arachnida, of which the most important members are the spiders and mites. Sambon (1922) classified the linguatulids in 13 genera and 43 species. At most five species have been found in man, the only ones occurring with noteworthy frequency as human parasites being *Porocephalus armillatus* (*Armillifer armillatus*) and *Linguatula serrata* (*L. rhinaria, L. denticulata*). *P. armillatus* is found in tropical Africa; its adult form is a parasite in snakes, in which it inhabits the air-passages and lungs. *L. serrata* has been reported from subtropical and temperate regions, particularly Europe; the adult parasite lives in the nasal fossae or paranasal sinuses of the dog or, less often, other animals (fox, wolf, horse, goat). The larvae of both species require a mammalian host in which to develop; infestation in the intermediate host is commonly known as pentastomiasis, the nymph stage of the parasite having formerly been thought to be a distinct species, to which the name *Pentastomum* was given because the curved claw

* [The authors' statement, which they have fully substantiated in a letter to the editorial office, that there are no previously published records of pentastomiasis in this country, deserves further comment since there can be little doubt that such infestation has occurred on a limited scale. The post-mortem records at the London Hospital yield a total of 38 cases in the years 1907 to 1942 inclusive, in which one or more pentastoma cysts were noted in the liver. Most of these were observed before the first world war, the highest incidence being seven cases in 1913. Two only have been recorded since 1927. The cysts were usually calcified. Microscopic preparations are available of 10 non-calcified examples. In none of these is the preservation of the parasite as good as in the cases described in this paper, though in all other respects the appearances are similar. There is nothing to suggest that their presence excited any clinical symptoms.—EDITOR.]

on each of the four rudimentary limbs surrounding the mouth superficially resembles an oral orifice (Küchenmeister, 1857). The life-cycle of the two species is similar ; the parasites differ significantly only in their definitive hosts and in certain morphological details. Descriptions of these parasites and of their pathogenicity are given by a number of authors (Fischer, 1929 ; Brumpt, 1949), and here only brief mention need be made of some aspects of the biology of *L. serrata* relevant to human infestation by this species, which was first recorded in man by Zenker (1854).

Linguatula serrata.—The adult parasite has a worm-like body, somewhat flattened dorsiventrally and tapering from before backwards ; its outline is crenellated by the presence of the approximately 90 rings by which it is characterized. As the vermiform body has some resemblance to a tongue, the parasite acquired the name tongue-worm, by which it is popularly known. The male is white and measures 1.8 to 2 cm. in length and 0.3 cm. in its greatest breadth ; the female is greyish, 8 to 10 cm. long and 0.8 to 1 cm. across its widest part. The ova, which contain an embryo when laid, measure 90 by 70 μ ; they are shed in the nasal secretion of the host, and ingested by the intermediate host on contaminated grass or vegetables eaten raw. The acariform larva hatches in the alimentary tract and by means of its cephalic armature bores through the bowel-wall ; it is then carried in the lymphatic or blood stream to the mesenteric lymph-nodes or to the viscera, particularly the liver, lungs, spleen, and kidneys, where it encysts. The larva is said then to undergo about nine moultings during five to six months. The final larva, or nymph (the so-called *Pentastomum denticulatum*), is 0.5 to 0.6 cm. long and is curved crescent-wise, its ventral surface forming the concavity of the curve ; it has the same number of body-rings as the adult, and each ring has a series of slightly incurved, pointed toothlets near its caudad margin. Further development is dependent upon the parasite reaching the nose of its definitive host ; when ingested the larva may ascend from the stomach to the nose or be carried to the latter in vomitus ejected before it has been killed by the gastric secretion ; alternatively, the larva may enter the nose directly while the animal feeds. In the nose maturation is completed. The adult parasite sometimes causes nasal obstruction, but rarely any other disturbance ; occasionally mild, chronic rhinitis develops and causes slight discharge ; the latter or the simple obstruction may lead the animal to rub its muzzle with its paws or against the ground, thus helping in dissemination of the ova. According to Brumpt (1949) the adult parasite has only once been found in man.

Cattle, sheep, and various smaller herbivores, e.g., rabbits, are the usual intermediate hosts of *L. serrata* ; the presence of even massive infestation by the larvae appears not to cause any appreciable illness. Rats are sometimes infested and may be the source of infestation in dogs which do not have access to the tissues of the commoner intermediate hosts, e.g., on farms and in butchering establishments.

The incidence of human infestation by the larvae cannot be assessed, as few investigations concerning its occurrence have been recorded ; in Berlin, Max Koch (1906) found the larvae in 11.75% of 400 necropsies, Laengner (1906) in 3% of 500 necropsies, and Sonobe (1927) in 3.2% of 500 adult cadavers ; the figures for other parts of Germany and Europe are much lower (Sagredo, 1924 ; Brumpt, 1949). Usually only a single larva is found in each case, and the liver has been the site of

the majority of the examples recorded. Infestation is said to be particularly rarely found in children (Sonobe, 1927).

Case Reports

Case 1.—L. P. (United Birmingham Hospitals Reg. No. G.264685), a married woman aged 41, was admitted to the General Hospital, Birmingham, on November 18, 1948. She complained of flatulence and a feeling of fullness after meals, particularly after eating fatty food ; these symptoms were of two years' duration. For two months before admission she had been troubled by aching pain in the right hypochondrium, just below the costal margin ; the pain occurred after eating and on exertion, and had gradually become more noticeable. Her appetite had become poor, and she suffered from nausea, with vomiting after eating fats. There had been no loss of weight. Apart from these symptoms she was in good health, and worked actively as a district nurse ; her only previous illness was acute appendicitis, a year before the onset of the present symptoms. There was no family-history of illness.

The only clinical abnormality was slight tenderness in the right hypochondrium, in and near the gall-bladder area ; the old appendicectomy scar was healthy.

Investigations.—Radiological examination showed that the gall-bladder, stomach, and duodenum were normal. Bockus's biliary drainage test was negative. The urine was normal. Haematological examination gave the following results: erythrocytes 4.63 million per c.mm. ; haemoglobin 92% (Haldane), equivalent to 12.7 g. per 100 ml. ; leucocytes 5,500 per c.mm., of which neutrophil polymorphonuclears accounted for 66% (3,630), eosinophils 4.5% (247), basophils 0.5% (27), lymphocytes 19.5% (1,073), and monocytes, 9.5% (523). Serum chemistry showed: total proteins 7.75 g. per 100 ml., albumen 5.8 g., globulin 1.95 g. ; bilirubin 0.3 mg. per 100 ml. ; alkaline phosphatase 4 units (King and Armstrong) ; thymol turbidity test: 3 units (Maclagan) ; colloidal gold reaction: no precipitate. Hippuric acid excretion: 1.59 g. in one hour following 1.77 g. of sodium benzoate intravenously.

As medical treatment was proving of little value it was decided to perform a laparotomy. At operation (December 6, 1948) a slight inflammatory reaction in the serosa of the gall-bladder and adjacent liver was found, and a small greyish nodule on the surface of the liver in this region was excised ; cholecystectomy was performed.

Progress.—Convalescence was interrupted by repeated pulmonary embolism, which necessitated ligation of the right common iliac vein (January 14, 1949). The patient was discharged to a convalescent home on February 26, 1949, and her general condition slowly improved during the following months ; the dyspeptic symptoms gradually lessened, but were still troublesome when she was last seen, in April, 1950.

Following the discovery of a linguatula larva in the liver, the stools and sputum were repeatedly examined for parasites, with negative results. From February to December, 1949, she was treated empirically by courses of neoarsphenamine. A radiograph of her liver in January, 1950, showed no evidence of linguatula nodules ; the lung-fields were clear and the diaphragmatic movements, stomach, and duodenum radiologically normal.

Enquiry at the time when the parasite was found revealed that the patient owned an Alsatian wolf-hound ; unfortunately the animal was killed and the carcass destroyed before it could be examined. No signs of nasal disease or of any parasites had ever been observed in it.

Histological Examination.—The excised portion of liver, after fixation in formol-saline solution (4% formaldehyde), measured 8 by 6 mm. on its serosal surface and 4 mm. in depth ; an ill-defined grey area, 4 mm. in diameter, could be seen through

the serosa. The specimen was imbedded entire and a representative section cut. When an encysted parasite (Fig. 1) was recognized in the latter the rest of the block was sectioned serially. The parasite was identified as the larva of *Linguatula serrata* by the characteristic circumoral hooks, the closely placed body-rings with their toothlets (Fig. 2) and the internal structure, which was perfectly preserved. The cyst was lined by a very thin, smooth membrane of homogeneous, refractile, eosinophil material separating its cavity from the host-tissues. The cyst was surrounded by a granulomatous reaction (Fig. 2); a narrow zone of epithelioid histiocytes adjoined the membranous cyst-wall, closely moulded to which were occasional "foreign-body" giant-cells; many eosinophil leucocytes were present between the epithelioid cells, and a smaller number of lymphocytes. The epithelioid-cell zone was surrounded by a considerably wider zone of dense lymphocytic infiltration in which occasional large lymphoid follicles with prominent Flemming centres had formed. There was no fibrosis or necrosis. Serial sections demonstrated the characteristic C-shape of the

FIG. 1.—Case 1: Encysted larva of *Linguatula serrata* beneath serosa of liver. (Haematoxylin-eosin, × 80.)

FIG. 2.—Case 1: Granulomatous reaction around encysted larva. (Haematoxylin-eosin, × 400.)

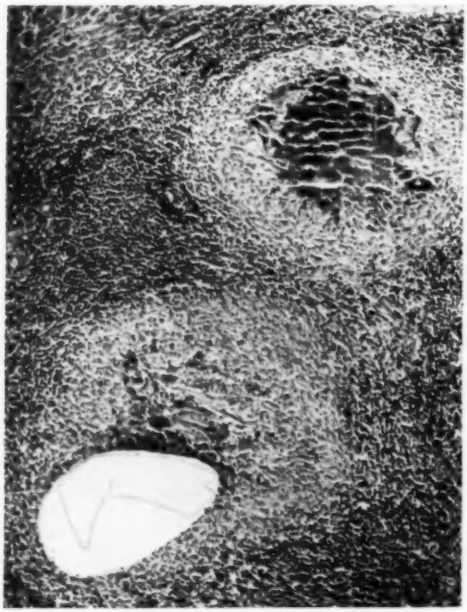

FIG. 3.—Case 1: Pseudotubercle-formation around remnants of cuticle shed by larva during moulting. (Haematoxylin-eosin, × 96.)

larva and the cyst containing it; in the granulomatous tissue filling the concavity of this curve groups of narrow, homogeneous, refractile, intensely eosinophil structures were present, arranged in palisade fashion and surrounded by macrophages, some of which were multinucleated. In places definite pseudotubercles had formed around these structures, which appeared to be remnants of a cuticle shed by the larva during moulting (Fig. 3).

The hepatic lobules in the vicinity of the granuloma were slightly distorted but otherwise normal. The small portal tracts were heavily infiltrated by lymphocytes, amongst which eosinophil leucocytes were present in considerable numbers. The granuloma reached to the surface of the liver and corresponded with the grey area noted macroscopically; the overlying Glisson's capsule was normal.

Sections of the gall-bladder showed no abnormality.

Case 2.—At necropsy on a girl, aged 17 years, who had died with pulmonary tuberculosis complicated by tuberculous ileitis and tuberculous meningitis, several firm, grey nodules were found scattered throughout the liver, the majority just deep to its capsule. The nodules measured 2 to 3 mm. in diameter, and were thought to be tuberculous lesions undergoing fibrosis under the influence of streptomycin therapy. A portion of liver containing one nodule was fixed in formol-saline solution and paraffin sections examined; unfortunately, serial sections through the whole block were not prepared, contrary to intention, and a considerable part of the lesion was therefore lost.

Histology.—The lesion consisted of two broad rings of practically acellular, partly hyalinized, collagenous fibrous tissue, each surrounding a central mass of amorphous material in which remnants of a parasite were present (Fig. 4). There was little intermingling of the fibrous tissue at the point of contact between the two rings, the angles between which were filled by loose-textured connective tissue, densely infiltrated by lymphocytes. The periphery of the lesion was sharply demarcated from the surrounding liver-tissue, which showed no abnormality. At the surface of the liver the fibrous rings merged into the deep surface of Glisson's capsule, which was not thickened. No eosinophil leucocytes were seen in any part of the tissues.

The parasite was identified as a larva of *L. serrata* by the characteristic ring-markings and toothlets on the cuticle, which was still easily recognizable (Fig. 5). The rest of the larva had partly disintegrated into amorphous detritus and partly become impregnated with calcium salts. It is probable that the two nodules seen in the sections

represented in fact one lesion; the larva of *L. serrata* is crescent-shaped, and the sections appeared to have been cut through the limbs of the crescent near their ends.

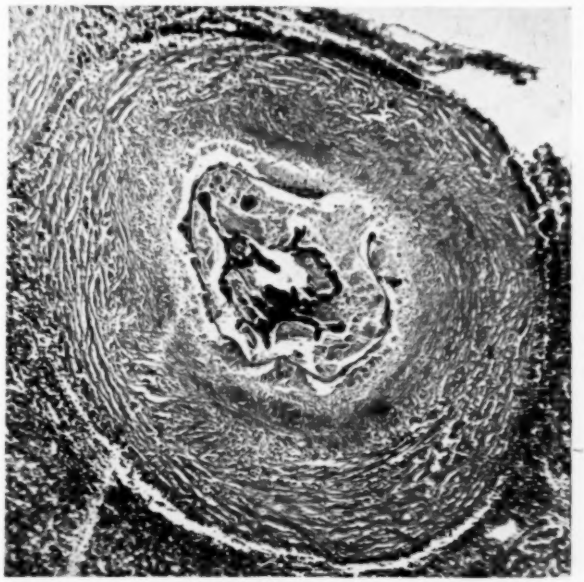

FIG. 4

Discussion

The findings in Case 2 are typical of those described in the majority of previous cases; in most of the latter the parasite had long since died, partially disintegrated and begun to calcify, while the end-result of the reaction of the host's tissues was represented by a dense, fibrous capsule around the remnants of the larva, with little or no remaining sign of active inflammation. In Case 1, on the other hand, the parasite was perfectly preserved and the granulomatous reaction around the cyst was in an early and active stage, with no demonstrable fibrosis.

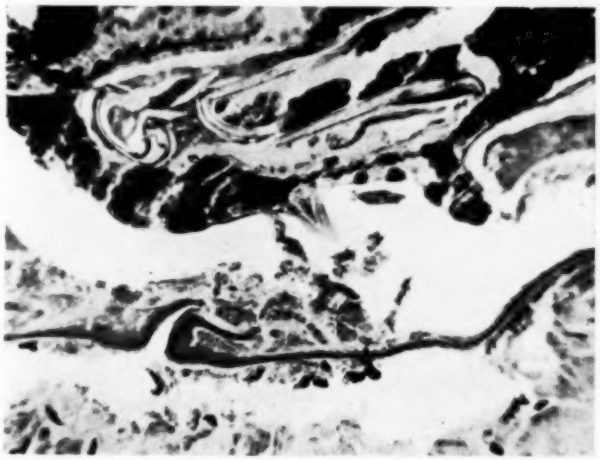

FIG. 5

FIG. 4.—Case 2: "Linguatula nodule" consisting of an outer zone of fibrous tissue surrounding amorphous material in which remnants of the parasite are seen. (Haematoxylin and van Gieson's stain, × 74.)

FIG. 5.—Case 2: Remains of larva, with still recognizable cuticular toothlets. (Haematoxylin and van Gieson's stain, × 400.)

Sonobe (1927) described a "nutritive knob or cone" (*Ernährungshöcker* or *-zapfen*) formed by the host's connective tissues filling the concavity on the ventral aspect of the encysted parasite, and from which the latter was said to obtain its nutrition. It seems more probable that the larva obtains its nourishment from the fluid in the cyst surrounding it and not by attaching itself to a particular region of the wall of the cyst to suck "nutrient juices" from the host's tissues. The appearances of the granulomatous tissue in contact with the cyst in Case 1 were uniform in all areas, and the membrane lining the cyst was everywhere intact. At the side corresponding with the concave (ventral) aspect of the larva in the same case, compressed remnants of a previously discarded cuticle were found far out towards the periphery of the inflammatory zone around the cyst; these remnants, which had provoked a typical foreign-body reaction, were in the situation described by Koch (1906) as characteristic.

The inflammatory reaction in Case 1 represents an early stage in the process by which the tissues wall off the larva by producing the dense fibrous capsule which is seen in cases of longer duration, and gives the characteristic naked-eye appearance and consistency to the so-called "linguatula nodules." The dead larva ultimately degenerates and the cyst collapses; calcification follows, and the nodules may become visible radiologically (Saupe, 1930). The greatest dimension of the lesion is 3 to 6 mm., rarely more.

Only one case has been reported in which the presence of the larva of *L. serrata* has been suggested as the cause of injury to the tissues of greater extent than the purely local inflammation around the encysted parasite. Sagredo (1924) found a larva in a haemorrhagic lesion in one lung of a youth who had died of encephalitis lethargica; in addition to haemorrhage there was some destruction and leucocytic infiltration of the lung-tissue, attributed by Sagredo to migration of the larva. Sonobe (1927) gives convincing reasons why the pulmonary lesion in Sagredo's case cannot be accepted as due to the presence of the parasite.

In another case (Roy and Ganguly, 1940) it was suggested that larvae of *L. serrata*, coughed and sneezed up by a woman whose main complaint was of pain over the frontal sinuses, were causally related to the symptoms.

In Case 1 of the present paper it is not possible to go farther than to suggest the possibility that the aggravation of the patient's symptoms and the pain in the right hypochondrium during the two months before her admission to hospital may have been caused by the infestation; how heavy the latter was is unknown. No other evidence of the presence of parasites was found, and attempts to demonstrate calcified linguatula nodules radiologically have so far been unsuccessful.

The source of the infestation remains unknown in each of our cases. No information about the prevalence of linguatuliasis in dogs in Britain is available; the absence of reports on this subject suggests that it is rare.

We have found no reference in medical or veterinary literature to methods of treatment of infestation by these larvae. There is no evidence that the courses of neoarsphenamine given in Case 1 played any part in the slow abatement of the patient's symptoms.

It is of interest to note that infestation by the larva ("*Pentastomum constrictum*") of the tropical linguatulid *Porocephalus armillatus* has on occasion been so heavy

in man as to cause illness and even death (Cannon, 1942). In contrast with this, there is, up to now, nothing to suggest that infestation by larvae of *Linguatula serrata* is of any clinical importance. The laborious and time-consuming investigation of necropsy material required to detect cases of the latter would therefore yield results of academic value only. In the course of routine necropsy work in two of the United Birmingham hospitals the opportunity has been taken to search for linguatula nodules in the liver, spleen, kidneys, and mesentery. In 400 consecutive necropsies on adults a positive finding was made only once (Case 2, above).

Summary

Two cases of human infestation by the larval form of *Linguatula serrata*, one of the so-called tongue-worms, are described.

In one patient the infestation, diagnosed by identifying the larva in a portion of liver removed during cholecystectomy, may have played some part in causing her symptoms. In this case treatment with neoarsphenamine was given without conclusive effect. The second case was discovered incidentally at necropsy, and accords with the usually accepted view that *Linguatula serrata* is not of practical importance in human pathology.

We should like to thank Dr. Ronald St. Johnston for permission to publish the case of his patient (Case 1), and Mr. Fauset Welsh for allowing us to refer to the notes of his findings at operation on this patient.

We are indebted to Mr. Fred Bradley, University of Birmingham, for the photomicrography, and to Miss S. P. Katt for much assistance.

References

Brumpt, E. (1949). *Précis de parasitologie*, 6th ed., p. 1060. Paris.
Cannon, D. A. (1942). *Ann. trop. Med. Parasit.*, **36**, 160.
Fischer, W. (1929). In *Handbuch der speziellen pathologischen Anatomie und Histologie*, ed. by Henke, F., and Lubarsch, O. Vol. 4, part 3, p. 703. Berlin.
Koch, M. (1906). Quoted *verbatim* by Sonobe (1927).
Küchenmeister, F. (1857). *On Animal and Vegetable Parasites of the Human Body*, trans. by Lankester, E. Vol. 2. London.
Laengner, H. (1906). *Zbl. Bakt., I. Orig.*, **40**, 368.
Roy, D. N., and Ganguly, S. K. (1940). *Indian med. Gaz.*, **75**, 478.
Sagredo, N. (1924). *Virchows Arch.*, **251**, 608.
Sambon, L. W. (1922). *J. trop. Med. Hyg.*, **25**, 188, 389.
Saupe, E. (1930). *Röntgenpraxis*, **2**, 401.
Sonobe, K. (1927). *Virchows Arch.*, **263**, 753.
Zenker, F. A. (1854). *Z. rat. Med.*, **5**, 212.

J. clin. Path. (1950), 3, 220.

GIANT CELL GRANULOMA ASSOCIATED WITH LESIONS RESEMBLING POLYARTERITIS NODOSA

BY

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The association of peculiar giant-cellular granulomatous lesions of the upper respiratory tract and the lungs or other organs with necrotizing vascular lesions resembling polyarteritis nodosa has been seldom reported. Klinger (1931) described one case; Rössle (1936) one case; Wegener (1939) three cases; Neumann (1940) four cases; Banowitch, Polayes, and Charet (1942) one case; Lindsay, Aggeler, and Lucia (1944) one case; Weinberg (1946) two cases; and Smith (1948) one case. The aetiology of the cases reported was obscure, but such lesions have been associated with various factors, for example, foreign serum, foreign serum and sulphonamide therapy, or sulphonamide therapy, especially sulphathiazole alone (Lederer and Rosenblatt, 1942; Rich, 1942a, 1942b; Rich and Gregory, 1943; Lichtenstein and Fox, 1946; Duff, More, and McMillan, 1946; van Rijssel and Meyler, 1949; Olesen and Myschetzky, 1949; and others). Rössle (1936) considered a rheumatic aetiology and Weinberg (1946) pointed out that similar granulomatous lesions of unknown aetiology, but not associated with polyarteritis nodosa, have been described as far back as 1906.

The salient features, according to Weinberg, were a history of long-standing infection of the upper respiratory tract with the development of ulceration of the nose, mouth, and trachea and with the final development of polyarteritis nodosa. Rössle's case showed involvement of the paranasal sinuses and the ear. Wegener's three cases followed a peculiar granulomatous rhinitis. Lindsay's case apparently first manifested itself as a painless depression of the bridge of the nose about a year before death due to polyarteritis nodosa. Also, one case of polyarteritis nodosa of the series reported by Davson, Ball, and Platt (1948), and that of Olesen and Myschetzky (1949), both had had a Caldwell-Luc operation performed for chronic nasal discharge and maxillary sinusitis some months before death from polyarteritis nodosa. We observed an example of this remarkable association, and the following is an account of the clinical picture, post-mortem findings, and some aspects of the pathogenesis.

Case Report

G. D., a married man aged 23, was seen on March 24, 1949, in the outpatient department of the Royal Ear, Nose, and Throat Hospital, London, complaining of increasing nasal obstruction for two months and nasal discharge for three months. The nasal

mucosa was intensely congested and granular, with a great deal of crusting in the vestibule. Radiographs showed opacity of both antra and a cloudy left frontal sinus. On April 7, 1949, the patient was seen again and a biopsy taken from the mucosa of the right inferior turbinate. This showed (Fig. 1) " fragments of granulation tissue with foreign body giant cells and eosinophils and some areas of necrosis. No evidence of malignant change." He was not seen again until April 27, 1949, when he was transferred from another hospital, where he had been admitted in the meantime, with a note stating that he had been treated with sulphadiazine and his condition had not improved.

On examination the patient was seen to be an ill-looking man with gingivitis, a large perforation in the cartilaginous septum, and crusting in both sides of his nose. Inspection of the ears showed injection of both drums posteriorly and oedema of the arytenoid region of the larynx. A right Caldwell-Luc operation was performed, and a portion of the antral mucosa removed for histological examination.

The patient had persistent slight fever after operation. On May 17 oedema of both lower lids was observed. On May 23 the patient was rather drowsy, vomiting greenish material, and had a very foul postnasal discharge. Oliguria was present. He passed two ounces of urine in which were blood and albumin on May 24. The blood pressure was 155/80 mm. Hg. On May 28 oedema of the ankles was increasing, and the patient was very drowsy. His nose was still full of crusts, and he passed little urine. His blood pressure was 170/80 mm. Hg, and on May 30 it was 170/90 mm. Hg.

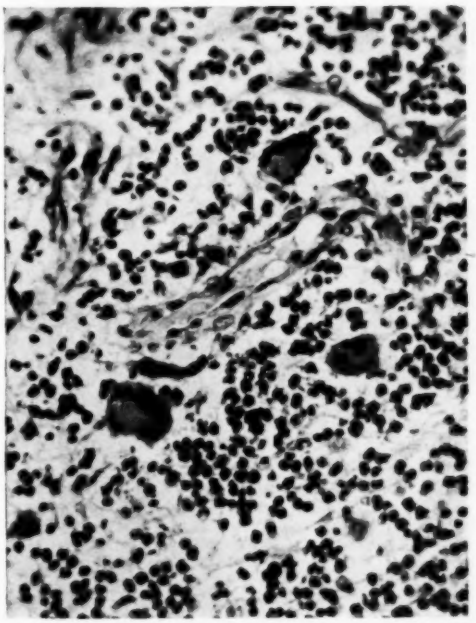


FIG. 1.—Photomicrograph of right inferior turbinate showing numerous giant cells and eosinophils. (Haematoxylin and eosin. × 285.)

The patient was treated with penicillin from April 29 to May 9, and from May 17 to May 30. The total amount given was 16,000,000 units. From May 21 " albucid " (sulphacetamide) was given four-hourly. Death took place on June 4, 1949.

Laboratory Data

Examination of biopsy material from the antrum (Dr. Lucas) showed no normal mucosa. It was replaced partly by granulation tissue and partly by fibrous tissue with some areas of haemorrhage and pus. Giant cells were numerous, especially in the granulation tissue. There was no evidence of specific inflammation or neoplastic change.

Bacteriological Findings.—Culture from the maxillary antrum (29.4.49) showed a mixed growth of *Streptococcus beta haemolyticus* (penicillin-sensitive) and *Staphylococcus*

pyogenes (penicillin-sensitive). On 23.5.49 *Staphylococcus pyogenes* (penicillin-resistant and streptomycin-sensitive) only was grown.

Blood culture (25.5.49) was sterile after seven days' incubation.

Blood Counts.—(30.4.49) White blood cells, 7,650; haemoglobin, 70% (Sahli); red blood cells, 3,300,000; polymorphs, 73.5%; lymphocytes, 23.5%; monocytes, 3%; eosinophils, not seen. On 24.5.49 white blood cells, 8,800; haemoglobin, 72%; red blood cells, 4,570,000. On 31.5.49 white blood cells, 31,800; and on 3.6.49 white blood cells, 27,300.

The Wassermann reaction was negative.

Urine (27.5.49).—Albumin present. Numerous red blood cells and granular casts seen in deposit. Culture sterile.

Blood Urea.—On 24.5.49 was 160 mg.%, and thereafter (28.5.49) 220 mg.%; (31.5.49) 260 mg.%; (3.6.49) 320 mg.%.

Cerebrospinal Fluid.—On 26.5.49 C.S.F. was clear. Pressure, 130 mm.; cells, 3; proteins, 10 mg.%; chlorides, 731 mg.%.

Necropsy Findings

Necropsy was performed six hours after death.

The body was that of a well-built, well-nourished, pale young man. There was some dry purulent discharge from the nose, and pitting oedema of both legs.

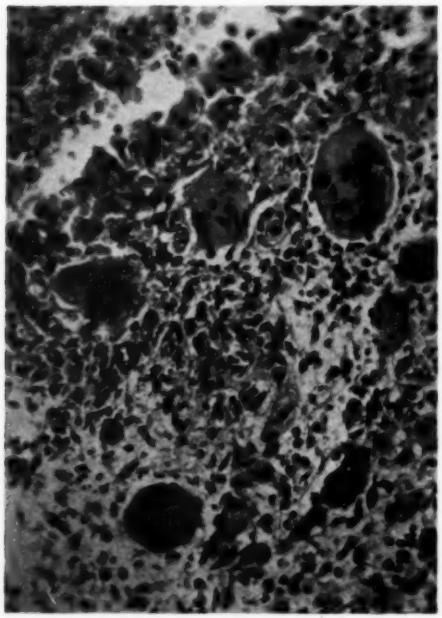


FIG. 2.—Photomicrograph of maxillary antrum showing granulation tissue with numerous multinucleated giant cells of the foreign-body type. (Haematoxylin and eosin. × 240.)

Skull.—Maxillary sinuses contained a great deal of purulent exudate. Ethmoids and frontal sinuses filled with thick creamy yellow purulent exudate. Middle ear was dry; the meninges dry and smooth. Brain, normal apart from pallor. Blood vessels normal.

Chest.—There were numerous shallow ulcers in the larynx and trachea, the largest about 2 cm. in diameter, of "mousebitten" appearance with irregular edges and necrotic bases, in the subglottic area.

About 200 ml. of clear fluid occupied each pleural cavity. Patchy, dark red, haemorrhagic consolidation in lower lobes of both lungs. On pressure great amounts of sanious fluid escaped from the cut surface. Frothy fluid in air passages. Some emphysema of upper lobes. The heart was normal apart from cloudy swelling.

Abdomen.—The peritoneal cavity contained about 4 pints of clear, straw-coloured fluid. The firm, lobular pattern of the liver was accentuated. The spleen (about 400 g.) was soft, and the cut surfaces revealed a hyperplastic pulp and prominent Malpighian bodies.

The kidneys were enlarged (about 250 g.). The capsule stripped easily, revealing smooth swollen surfaces peppered with numerous petechial haemorrhages. On section the glomeruli were very prominent and the cortex similarly contained petechiae. No infarcts were present.

The pelves, ureter, bladder, and prostate were normal, as also were the alimentary canal, thyroid, and adrenals.

FIG. 3.—Photomicrograph of emphysematous lung tissue showing necrotizing arteriole surrounded by pleomorphic inflammatory cells. (Haematoxylin and eosin. × 130.)

Microscopical Examination. — Sections were stained with haematoxylin and eosin, van Gieson, phosphotungstic acid-haematoxylin, Weigert's elastic stain, Gram, and Ziehl-Neelsen.

Sections from the maxillary antrum (Fig. 2) showed non-specific, highly vascular granulation tissue, containing numerous multinucleated giant cells of the foreign-body type often with bizarre arrangements of the nuclei and a foamy cytoplasm. There were numerous areas of necrosis infiltrated by polymorphonuclear leucocytes, and extensive haemorrhage in some areas. Some vessels appeared to be involved in the necrotic process, but it was not possible to assess the type of vascular lesion in this necrotic granulation tissue. Gram and Ziehl-Neelsen stains showed no organisms.

Sections of the purulent tissue from the frontal sinus showed mainly

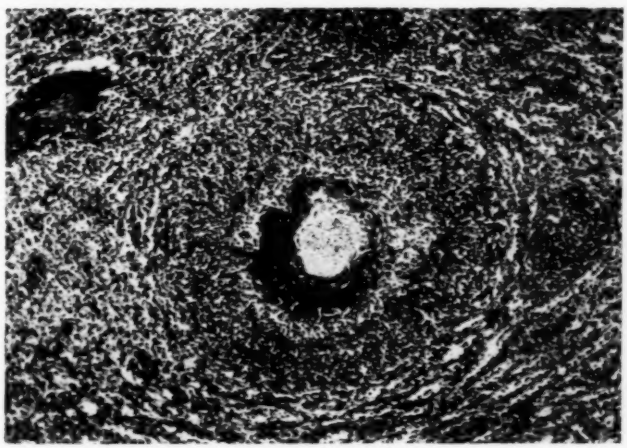


FIG. 4.—Marked fibrinoid necrosis of follicular arteriole in the spleen surrounded by a predominantly leucocytic exudate. (Haematoxylin and eosin. × 75.)

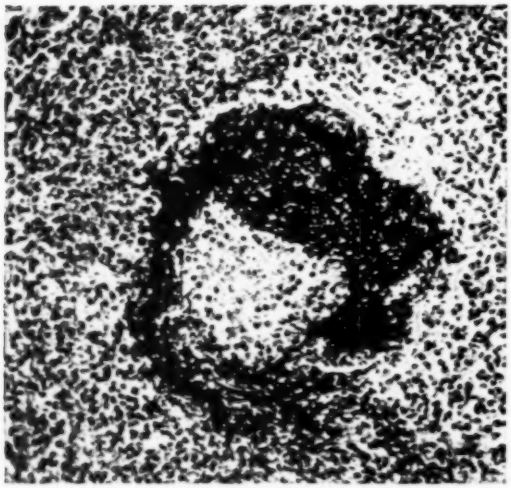


FIG. 5.—Fibrinoid necrosis of follicular arteriole in the spleen. (Phosphotungstic acid-haematoxylin. × 160.)

necrotic purulent exudate with a few large mononuclear cells.

The floor and edges of the large ulcer from the subglottic area were formed by a necrotizing granulation tissue consisting of numerous leucocytes, epithelioid cells, and a fair number of giant cells. There was some resemblance to a tuberculous ulcer, but no tubercle bacilli were found either in section or in cultures.

Lungs. — Sections from the lower lobe of the lungs showed widespread haemorrhage and collateral emphysema. In the emphysematous area there were vascular changes suggestive of polyarteritis nodosa (Fig. 3). The small necrotic arterioles were surrounded by a zone of polymorphs, lymphocytes, and a few eosinophils.

Spleen.—Sections of the spleen showed a very widespread lesion affecting particularly the follicular arterioles (Fig. 4) and small arteries and possibly damaging the veins as well. The fully developed lesions consisted of segmental or total fibrinoid necrosis of the

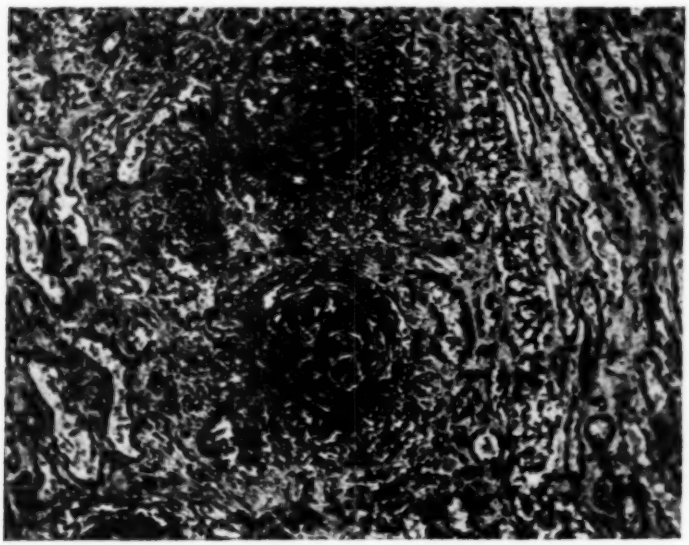


FIG. 6.—Necrotic glomeruli of the kidney with periglomerular infiltration, the parenchymatous elements separated by a young granulation tissue. (Haematoxylin and eosin. × 95.)

walls, well shown in sections stained with phosphotungstic acid-haematoxylin (Fig. 5). There was pleomorphic cellular exudation around the vessels, polymorphonuclear leucocytes predominating. The hyperplastic pulp contained numerous plasma cells, eosinophil and neutrophil leucocytes, and focal aggregations of leucocytes, some of which surrounded the trabeculae ("Trabeculitis" of More, McMillan, and Duff, 1946). The eosinophilic necrotic material of the vascular lesions seemed in places to be extending into the surrounding exudate.

Kidneys.—There was widespread damage throughout the cortex of the kidneys, and all the glomeruli were apparently affected (Fig. 6). In places some of the glomerular loops showed hyaline and fibrinoid necrosis and elsewhere all the loops were affected. The fibrinoid material formed thrombi, and in some glomeruli almost filled the capsular space (Fig. 7). There was marked proliferation of the epithelial cells of Bowman's capsule with epithelial crescent formation in many glomeruli. Some glomeruli were completely disorganized and replaced by granulation tissue, in which it was difficult to trace the remnants of the glomerular tufts. There was marked periglomerulitis, especially around some enlarged and distended glomeruli, which were surrounded by a wide zone of radially arranged cellular exudate, consisting of polymorphs, histiocytes, fibroblasts, and a moderate number of eosinophil leucocytes (Fig. 8). The parenchymatous elements of the cortex and medulla were separated by a young granulation tissue containing numerous eosinophils. Many of the tubules were dilated and contained cellular and granular casts. The majority of the afferent and other arterioles showed partial or total hyaline or fibrinoid necrosis. Only a round homogeneous ball- or ring-like structure of necrotic material remained in place of some arterioles which were surrounded by a radially arranged zone of neutrophil and eosinophil leucocytes and histiocytes (Fig. 9). The intralobar and arcuate arteries showed no necrosis, but in some instances there was perivascular infiltration with lymphocytes. An arcuate vein showed localized necrosis with associated leucocyte infiltration.

The liver showed some congestion and a few vascular lesions. A few branches of the portal vein were necrotic and surrounded by lymphocytes, monocytes, and numerous eosinophils.

The myocardium showed some fibrosis and a few small foci of mononuclear cells in the perivascular tissue. There was no endocarditis.

The carotid arteries and jugular veins showed no change.

The thyroid, tonsils, and submaxillary gland showed no change. The base of the tongue, however, showed a few necrotic vessels surrounded by granulomatous nodules among the mucous glands.

FIG. 7.—Extensive fibrinoid necrosis of glomerulus of the kidney. (Phosphotungstic acid-haematoxylin. × 145.)

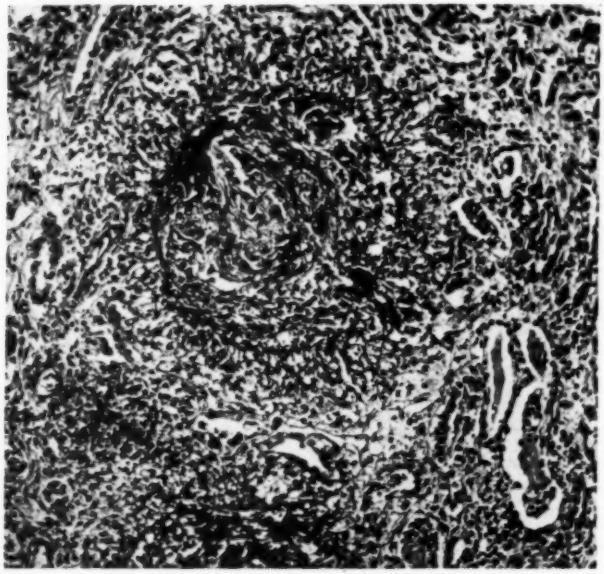
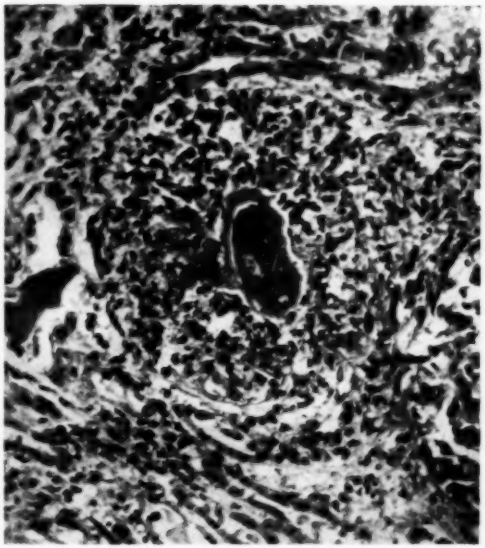


FIG. 8.—Disorganized distended glomerulus of the kidney with epithelial proliferation surrounded by a radially arranged zone of inflammatory cells. (Haematoxylin and eosin. × 120.)

FIG. 9.—Total fibrinoid necrosis of arteriole of kidney surrounded by exudate with numerous eosinophils. (Haematoxylin and eosin. × 235.)



Discussion

The case described presents the remarkable association of two types of lesions: (1) giant cell granulomata of the upper respiratory tract, and (2) widespread necrotizing vascular lesions resembling polyarteritis nodosa.

The condition of the nose and of the paranasal sinuses, the first apparent site of a giant-cellular granulomatous reaction encountered in some cases of polyarteritis, dominated the clinical picture in the beginning. This initial stage of chronic sinusitis, lasting about four to five months, was followed, as in other cases, by fatal renal failure and uraemia.

The kidneys showed the microscopic features of the rapidly progressive type I of nephritis (Ellis,

1942), namely, explosive lesions of the glomeruli, definite periglomerular inflammatory reaction, and inflammatory infiltration of the interstitial tissue. There was marked hyaline and fibrinoid necrosis of the glomerular tufts and arterioles.

There was also widespread necrotizing angiitis in other viscera, especially in the spleen and the lungs, but also in the liver.

The extra-renal vascular changes suggested polyarteritis nodosa, and the renal lesions were also consistent with the description of the kidney in the microscopic form of polyarteritis nodosa (Davson, Ball, and Platt, 1948 ; Davson and Platt, 1949). In a case like this it is, however, very difficult to draw an absolute distinction between the rapidly progressive type I of nephritis and polyarteritis nodosa (Group I of Davson *et al.*, 1948). In any case the renal and vascular lesions certainly are connected and two distinct pathogenetic factors may be considered: (1) Hypertension due to nephritis causing in turn the necrotizing vascular lesions ; (2) hypersensitivity as the underlying cause of both the renal and vascular changes primarily affecting the vascular system.

There is evidence (Smith and Zeek ; Zeek, Smith, and Weeter, 1948) to support the hypothesis that polyarteritis nodosa is associated with sharply rising hypertension and is not so much the result of hypersensitivity to known or unknown antigens. Numerous authors, however, believe that polyarteritis nodosa is a manifestation of the anaphylactic type of hypersensitivity (Rich, 1942a, 1942b ; Rich and Gregory, 1943).

In our case the blood pressure was never very high and only rose to 170/90 mm. Hg shortly before death from renal failure and uraemia. On the other hand there was a certain amount of morphological evidence that the lesion was of the kind described by Zeek *et al.* (1948) as " hypersensitivity angiitis," and attributed by them to hypersensitivity. These authors described a vascular lesion as " hypersensitivity angiitis " in seven cases of necrotizing arteritis in men which showed clinical evidence of hypersensitivity during life, usually to one of the sulphonamides. In distinction to the " classical " type of polyarteritis nodosa in men and rats, hypersensitivity angiitis affected only the small arterioles, in particular the follicular arterioles in the spleen. The lungs and liver were also affected. There was no evidence of healing and frequently the entire wall of the small vessels presented fibrinoid necrosis. There was also necrotizing glomerulitis characterized mainly by exudation and necrosis, with little evidence of the epithelial proliferation so marked in the kidneys of the case described.

The aetiology of the condition also remains obscure. Weinberg (1946) points to the history of antecedent chronic infection and bacterial hypersensitivity.

The organisms grown in our case during illness and from post-mortem specimens, namely *Staphylococcus pyogenes* and *Streptococcus beta haemolyticus*, are both capable of producing necrotizing glomerulitis and necrotizing vascular lesions.

In any case of polyarteritis nodosa complicating a disease the drug or drugs used should also be considered as causative agents (Pulvertaft, 1949). Our patient had been treated with sulphadiazine, sulphacetamide (" albucid "), and penicillin.

Hypersensitivity to sulphonamides, especially to sulphathiazole, has been incriminated in the causation of necrotizing vascular lesions resembling polyarteritis nodosa

(Hagemann and Blake, 1937 ; Erskine, 1939 ; French and Weller, 1942 ; Lederer and Rosenblatt, 1942 ; Merkel and Crawford, 1942 ; Rich, 1942a, 1942b ; Lichtenstein and Fox, 1946 ; van Rijssel and Meyler, 1948 ; Olesen and Myschetzky, 1949 ; and others).

Duff *et al.* (1946) studied a series of 375 necropsies in which it was known that considerable doses of sulphonamides had been given during life. In 22 cases lesions attributable to the sulphonamide treatment were found, the most frequent lesion being a granulomatous reaction encountered in 13 out of the 22 cases. In seven cases an acute necrotizing arteritis was found whilst four out of 22 cases had both lesions. These authors believe that the granulomata belong to, and are part of, the vascular disease, and Lindsay *et al.* (1944) suggest that polyarteritis nodosa be considered in any granulomatous lesion of unknown aetiology.

In our case we assume that the giant-cell granulomata had been present for three to five months and histologically confirmed in the maxillary antrum about 10 weeks before death. It would thus appear that the granulomata were the first manifestation of the vascular disease possibly due to hypersensitivity to an unknown agent.

Summary

A case is described of giant-cell granulomata of unknown aetiology in the paranasal sinuses associated with necrotizing vascular lesions resembling polyarteritis nodosa terminating in renal failure.

The vascular lesions were widespread, affecting the arterioles of the viscera, including the kidneys and the spleen, and presented the morphological features of " hypersensitivity angiitis " as described by Zeek *et al.* (1948).

Evidence is presented that the paranasal sinuses or the nose can be the first site of the peculiar granulomatous reaction encountered in some cases of polyarteritis nodosa.

Our thanks are due to Dr. Lucas for his interest and helpful encouragement ; to Mr. E. S. Bird, F.I.M.L.T., for the sections ; and to Mr. E. V. Willmott, F.R.P.S., for the photomicrographs.

REFERENCES

Banowitch, M. M., Polayes, S. H., and Charet, R. (1942). *Ann. intern. Med.*, **16**, 1149.
Davson, J., Ball, J., and Platt, R. (1948). *Quart. J. Med. n.s.*, **17**, 175.
—— and Platt, R. (1949). Ibid., **18**, 149.
Duff, G. L., More, R. H., and McMillan, G. C. (1946). *Amer. J. Path.*, **22**, 646.
Ellis, A. (1942). *Lancet*, **1**, 72.
Erskine, D. (1939). *Brit. J. vener. Dis.*, **15**, 260.
French, A. J., and Weller, C. V. (1942). *Amer. J. Path.*, **18**, 109.
Hageman, P. O., and Blake, F. G. (1937). *J. Amer. med. Ass.*, **109**, 642.
Klinger, H. (1931). *Frankfurt. Z. Path.*, **42**, 455.
Lederer, M., and Rosenblatt, P. (1942). *J. Amer. med. Ass.*, **119**, 8.
Lichtenstein, L., and Fox, L. J. (1946). *Amer. J. Path.*, **22**, 665.
Lindsay, S., Aggeler, P. M., and Lucia, S. P. (1944). Ibid., **20**, 1057.
Merkel, W. C., and Crawford, R. C. (1942). *J. Amer. med. Ass.*, **119**, 770.
More, R. H., McMillan, G. C., and Duff, G. L. (1946). *Amer. J. Path.*, **22**, 703.
Neumann, R. (1940). *Arch. Path. Anat.*, **306**, 389.
Olesen, H., and Myschetzky, A. (1949). *Acta path. microbiol. scand.*, **26**, 142.
Pulvertaft, R. J. (1949). *Proc. R. Soc. Med.*, **42**, 502.

Rich, A. R. (1942a). *Bull. Johns Hopk. Hosp.*, **71**, 123.
—— (1942b). Ibid., **71**, 375.
—— and Gregory, J. E. (1943). Ibid., **72**, 65.
Rijssel, T. G. van, and Meyler, L. (1948). *Acta med. scand.*, **132**, 251.
Rössle, R. (1936–7). *Arch. Ohr.-Nas.-, u. Kehlk-Heilk.*, **142**, 193.
Smith, C. C., and Zeek, P. M. (1947). *Amer. J. Path.*, **23**, 147.
Smith, J. F. (1948). *J. Path. Bact.*, **60**, 489.
Wegener, F. (1939). *Beitr. path. Anat.*, **102**, 36.
Weinberg, T. (1946). *Amer. J. clin. Path.*, **16**, 784.
Zeek, P. M., Smith, C. C., and Weeter, J. C. (1948). *Amer. J. Path.*, **24**, 889.

J. clin. Path. (1950), 3, 230.

THE BONE MARROW IN HUTCHISON'S SYNDROME

BY

A. PINEY,* J. MALLARMÉ,† AND M. S. ROSS‡

(RECEIVED FOR PUBLICATION MAY 2, 1950)

Since Hutchison's now classical paper "On Suprarenal Carcinoma in Children with Metastases in the Skull" appeared in 1907, the syndrome, which now bears his name, has frequently been observed and recorded.

The salient features are the development of malaise and some degree of pallor in a previously healthy child, followed by left-sided proptosis, due to metastases in the orbit, together with signs of other secondary deposits in the cranial bones. The primary tumour, which has the histological structure of a neuroblastoma, with more or less well-developed "rosettes," is situated in the left suprarenal gland, where it may form a mass sufficiently large to be palpable. Less commonly, pains in the bones are the presenting symptom, while, occasionally, severe anaemia is an early sign, as in the case to be recorded here.

Case Report

M. K., a girl aged 6½ years, complained of pain in the right thigh during April, 1949. A radiograph taken at this time is said to have shown some diffuse rarefaction of the upper third of the right femur, and the leg was therefore put in plaster. Slight, left-sided exophthalmos developed, but, as an ophthalmologist could find no abnormality other than some enlargement of tarsal glands, little attention was at first paid to this. Then the child became obviously ill, with pallor and anorexia, and was transferred to the care of one of us in Paris (September, 1949).

At this time, she was a fairly well-nourished but pale child, with no detectable enlargement of the liver, spleen, or glands, but with fairly severe left-sided proptosis. There were no complaints of pain, and no tenderness of any of the bones could be detected.

The blood picture was that of a simple hypochromic anaemia.

Red corpuscles	2,600,000 per c.mm.
Haemoglobin	40%
Leucocytes	3,700 per c.mm.
Neutrophils	49%
Eosinophils	7%
Monocytes	2%
Lymphocytes ...	42%

No abnormal red or white cells were seen.

A second blood count, a week later, showed little change, but it was stated that there were 3% leucoblasts. Unfortunately this film was not preserved.

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X-ray examination of the skeleton revealed widespread changes.

The pelvis and upper ends of the femora (Fig. 1) showed increased density and thickening of the bony trabeculae, with complete disorganization of the normal structure. There were some clear islets and some clear bands, which ran parallel with the

FIG. 1.—Radiograph of pelvis and upper ends of femora.

cortex and also with the epiphyseal cartilages. On the left side, the cortex of the right iliac bone seems to have been penetrated by actively growing tissue.

The cranium (Fig. 2) showed the presence of very small clear islets, more or less round in outline, but sometimes confluent. These were most numerous near the vertex but were scattered throughout the cranial vault. There was also dense opacity of the left maxillary sinus together with disappearance of the floor of the left orbit.

A sternal puncture was performed on September 17, 1949. Very little haemopoietic tissue was obtained, but the material was highly cellular, the overwhelming majority of the cells being of approximately the same type. They were reported as follows:

"Reticular cells, almost all undifferentiated, except for a few of leucoblastic type. Diagnosis: reticulo-leucosarcomatosis."

More detailed examination showed that the cells were not, in fact, all exactly alike. The majority were large, with finely stippled nuclei and very indistinct cytoplasmic outlines so that, where the cells lay close together, there was a syncytial appearance. In a few of the nuclei, a vacuole with a central dot of chromatin could be seen.

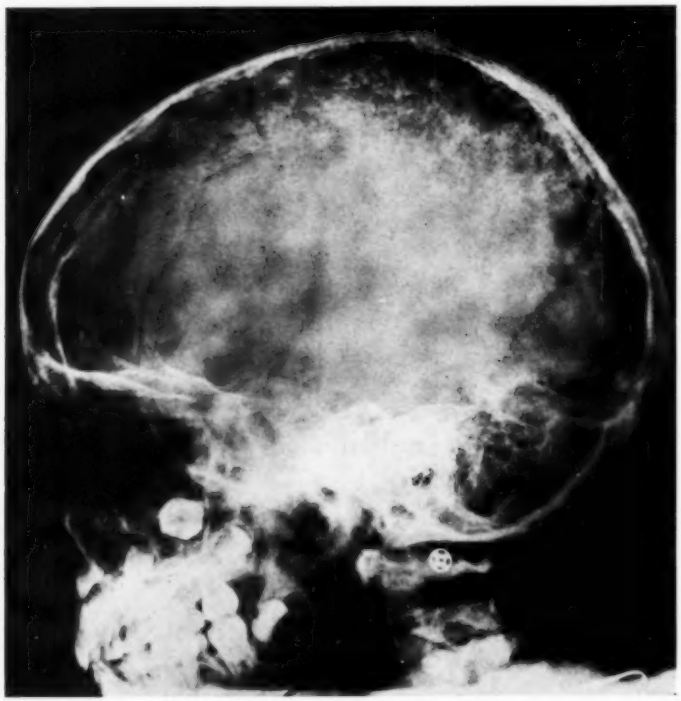

FIG. 2.—Radiograph of skull showing diffuse *pilzähnliche Metastasenbildung*.

In addition, a number of extremely degenerate elements were found, usually at the periphery of the masses (Fig. 3).

On October 8, 1949, the child was in a very poor condition, there was slight fever, and well marked left-sided exophthalmos, which was said to have improved considerably as a result of radiotherapy. There was no enlargement of liver, spleen, or glands, and no masses could be palpated in the abdomen. The bones were neither painful nor tender. There were no mucosal or cutaneous haemorrhages, although the child was strikingly pale.

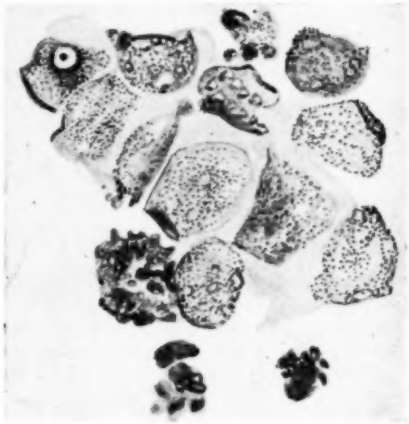

FIG. 3.—Drawing of a mass of cells from the marrow (16.9.49) showing the syncytial arrangement and one cell with a nuclear vacuole and several other degenerate elements.

A blood count on October 22, 1949, showed:

Red corpuscles	2,750,000 per c.mm.
Haemoglobin	60%
Leucocytes	2,600 per c.mm.
Neutrophils (myelocytes 3%; polymorphs 56%)	59%
Eosinophils	2%
Monocytes	4%
Lymphocytes ...	35%

2 normoblasts per 100 white cells.

On October 23, 1949, the child was brought to England. She was now extremely ill, and the proptosis was very great.

A sternal puncture was performed, and a very remarkable picture was seen. The myelogram had the following composition:

Polymorphs	24.0%
Lymphocytes	2.5%
Eosinophils	0.5%
Reticulum cells	72.0%
Normoblasts	1.0%

The reticulum cells occurred in large sheets and greatly predominated. Some of these cells showed active phagocytosis of leucocytes and red corpuscles (Fig. 4).

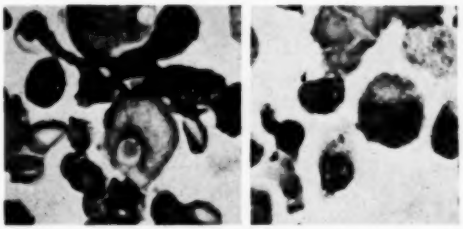

FIG. 4.—Phagocytic cells from marrow film: on the left a cell containing an ingested red corpuscle in its cytoplasm, and on the right one containing a degenerate polymorph.

The abnormal cells found in the marrow did not all present the same type of arrangement. Thus, there were groups of cells in which the individual elements were so closely packed that individual cell-boundaries could not be distinguished. Secondly, there were some more or less isolated cells with slightly stippled nuclei and rather indefinite cytoplasm. Some of these contained debris, but a similar appearance was also seen in a considerable proportion of the cells forming syncytial masses.

Not all the isolated cells showed cytoplasmic inclusions. Some appeared to be rather more highly differentiated. Some had rounded nuclei while others showed elongation of the whole cell body. Finally, there was a considerable admixture of cells showing advanced degeneration with fragmentation of the nuclei.

Between October 28 and November 7, 1949, four injections of nitrogen mustard (5 mg. in all) were given. The exophthalmos almost disappeared, while the general condition also improved, partly, no doubt, as the result of a small transfusion.

A sternal puncture on November 28, 1949, showed the following:

Polymorphs	2.0%
Lymphocytes	0.5%
Eosinophils	0.5%
Reticulum cells	96.0%
In mitosis	1.0%

"Many of the tumour cells are markedly degenerate. Phagocytic activity is still evident, but not so marked as in the previous specimen."

The syncytial masses were larger than those seen in the earlier films (Fig. 5), while more of the constituent cells had become somewhat elongated. Unfortunately, the amounts of marrow obtained on each occasion were very small, so that there was never enough for histological examination. In some of the areas in which the cells formed syncytial masses there was some indication of fibril formation (Fig. 6).

One attempt was made to obtain marrow by open operation on the iliac crest, but the specimen was too crushed to be of any diagnostic value.

The child went rapidly downhill, and died on December 29, 1949.

Necropsy

The findings at necropsy were as follows.

The body was that of a pale child, with extreme left-sided exophthalmos.

One slightly enlarged gland was found in the left upper cervical region, with an almost diffluent, pinkish-grey cut surface.

On the inner surface of the right ramus of the mandible there was a raised mass about 3 cm. in diameter covered by a thin layer of "crackling" bone and containing red pulpy material.

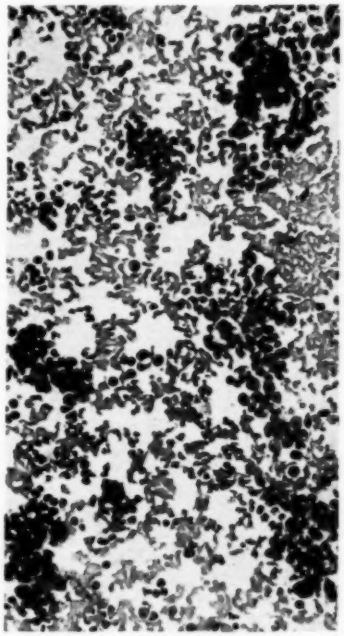

FIG. 5.—Photomicrograph of marrow film (× 125) showing agglomeration of the abnormal cells into mosaic-like groups.

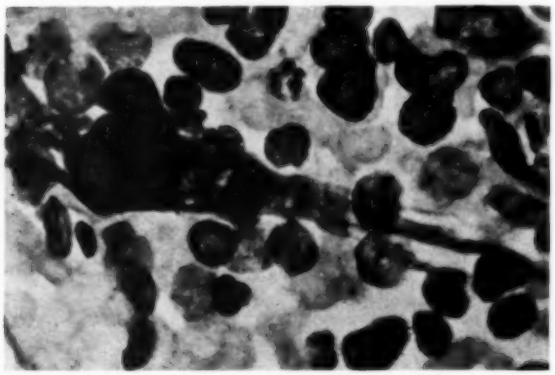

FIG. 6.—Fibrils in the substance of a syncytial mass are seen. (× 900.) Cells from sternal puncture.

There was one enlarged gland adjacent to the right margin of the thymus.

In the abdomen, there was a mass, 7 cm. in diameter, replacing the left suprarenal. This was covered with a thin capsule, and had a red, pulpy, haemorrhagic cut surface. There were several smaller similar nodules lying between the main tumour and the kidney (Fig. 7).

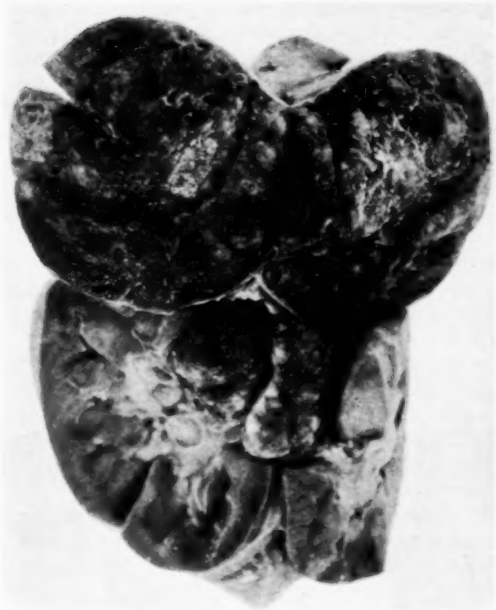

FIG. 7.—The left-sided suprarenal tumour.

The external surface of the calvarium showed a few dark areas where the cortex of the bone was extremely thin, and broke on pressure with the knife. The inner surface was covered by a sheet of plum-coloured material which had eroded the inner surface of the bone, and was adherent to the outer surface of the dura mater. There was a plum-coloured nodule situated in, and distending, the substance of the left petrous temporal bone. A large mass of haemorrhagic material filled the left orbital cavity, and was covered by a thin layer of bone in the roof of the left orbit, through which the colour of the underlying tumour was visible.

The only bones examined were the skull, sternum, vertebrae, and the left femur. These all contained red tissue, with, in places, more deeply plum-coloured areas, where also the overlying cortex was thinned.

Discussion

The post-mortem findings made it clear that the original diagnosis of reticuloleucosarcomatosis was incorrect, and it seemed certain that this case was an example of Hutchison's syndrome, due to a neuroblastoma of the left suprarenal gland.

Histologically, this diagnosis was confirmed, although, as is common in these cases, the characteristic "rosettes" were not found in all the neoplastic infiltrations.

The main tumour in the left suprarenal gland showed sheets of darkly staining round or polygonal cells. There were also fairly large haemorrhagic areas in and near which the darkly staining cells could be seen to form

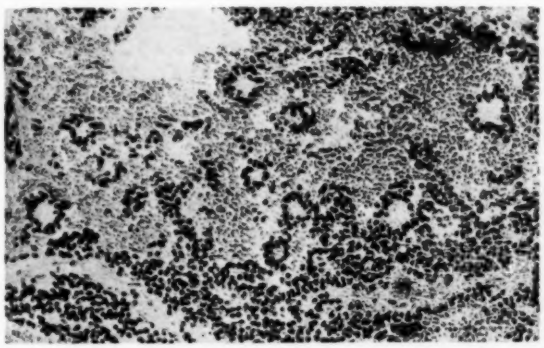

FIG. 8—Section of main suprarenal tumour showing well-marked formation of typical rosettes. (× 130.)

distinct rosettes (Fig. 8). It was, in fact, almost only in the main tumour that rosettes could be recognized: elsewhere the tumour cells formed irregular trabeculae. In a few places, e.g., the mandible, tumour cells could be seen lying in vascular channels, a clear indication of the main method of spread of the growth.

Search of the literature has revealed that this case is not unique, although only a few in which the marrow has been examined during life have been recorded.

In the case of Vogel and Bassen (1939): "The normal elements of the marrow were greatly depleted. The abnormal cells were not of the haematic series. They varied in size but were generally somewhat larger than myelocytes. They occurred in clumps, and surrounding many of them was a peculiar loose greyish cytoplasm containing fibrils." There is no mention of the presence of phagocytic cells, but, in their general discussion, the authors say that "ingested debris is frequently found in the cytoplasm of the reticulum cells." No radiographs are reproduced, but it is said that there were areas of rarefaction in the skull and long bones.

The case recorded in great detail by de Weerdt (1939) was rather different, inasmuch as the primary growth was in the coccygeal body. Marrow films contained a majority of small cells, which are said to have had a nuclear structure identical with that of haemocytoblasts. There is no reference to their being collected into groups, or to the presence of phagocytic cells. In this case, as in that of Vogel and Bassen, the diagnosis of neuroblastoma was not made during life.

Kato and Wachter (1938) found "clusters of unusually immature cells relatively large in dimensions and forming syncytial masses simulating a mosaic pattern." These elements are illustrated on a coloured plate, which closely resembles Fig. 5 of the present communication. There is no reference to the presence of reticulum cells. Radiologically, there was "a granular pattern in the parietal bones" and "questionable slight diastasis of the longitudinal suture." The diagnosis was not made until necropsy, but the authors briefly refer to a second case, in which sternal puncture showed "the presence of small aggregations of neuroblasts, identical with those seen in the first case." This is probably the first record of a diagnosis of this disease having been made by marrow puncture.

The report of Hooft and Compernolle (1942) contains some descriptions which are almost completely applicable to the present case. Thus, radiologically, the skull bones showed diffusely infiltrating metastases, which are compared with the appearance one would expect from the infiltration of a fungus (*pilzähnliche Metastasenbildung*). Examination of Fig. 2 shows how accurate is this vivid comparison. The authors assert that it is this type of metastatic infiltration that brings about the diastasis of the sutures, but this cannot always be true, because we found the fungus-like infiltrations without, however, any opening of the sutures. Similarly, the proptosis in our case was due to definite tumour-formation in the orbit, although Hooft and Compernolle attribute its occurrence to the *pilzähnliche Metastasenbildung*. A photomicrograph shows the mosaic-like arrangement of the tumour cells in the marrow, but no phagocytic reticulum cells are either shown or mentioned.

Albertini and Willi (1938) were among those who failed to make a diagnosis during life. But their report is of special interest, because they allege that abnormal cells, which they think were neoplastic elements, were found in the peripheral blood. In the present case, "leucoblasts" were said to have been found in the blood on one occasion, but the report of Albertini and Willi is not at all convincing,

and certainly the photomicrographs which purport to show the tumour cells in the blood are not such as to suggest anything more than the presence of rather atypical large lymphocytes. Their description of the radiographic appearances in the skull makes use of a comparison with the damage done by moths (*Mottenfrassartig*), but, certainly, in the present case the appearances are much more like those of a diffuse fungoid growth. The radiographic changes in the ilium and femora appear to have been very much like those we found.

Fanconi (1943) makes the surprising statement that, in Zürich during recent years, neuroblastomatosis has been as common as leukaemia. He briefly records three cases, in one of which marrow-puncture revealed the presence of the characteristic groups of tumour cells (*typische Tumorzellnester*).

Patschadji (1941) recorded his observations on the radiosensitivity of neuroblastoma cells, and found it to be very great. From the present point of view, a very interesting fact is that he mentions the finding of the characteristic marrow changes in four out of his five cases. He states that the syncytia consist of large polymorphous cells with little cytoplasm, lying so closely packed that, in places, their boundaries cannot be distinguished. Radiologically, the changes in the skull were of the moth-eaten rather than the diffuse " fungus " type.

The paper by Landolt (1946) is, haematologically at least, the most important one that has been published, although it gives little clinical or radiological information. Landolt emphasizes the significance of the mosaic-like arrangement of the cells in the marrow, whereas, he states, it is never found in sarcomatous metastases. He has, however, seen a somewhat similar arrangement of cells in cases of secondary carcinoma of the marrow.

The chromatin of the neuroblastoma cells, as seen in marrow films, is very delicate, and its structure is often impossible to detect. The cytoplasm is dirty-grey, sometimes with vacuoles, and often with very indistinct boundaries. If, however, one of the cells is found lying free, the cytoplasm can be seen to form a narrow zone with fairly distinct edges. When these neoplastic cells are plentiful in the marrow, macrophages are also numerous, although, according to Landolt, this is unusual in association with other marrow metastases. It is interesting to note that the number of reticulum cells (macrophages) was found to increase considerably after a week's radiotherapy, many showing signs of active phagocytosis of tumour-cell debris.

There appears to be no doubt that the present case is one of neuroblastomatosis and that in its general features it resembles the similar cases that have been published. Even so, a point of great interest and importance emerges, inasmuch as some other authors have not mentioned the presence of any phagocytic cells in the vicinity of the neuroblastic elements in the marrow, while others, especially Landolt, have laid emphasis on their presence.

At first we were inclined to agree with Landolt's view that the neuroblastoma cells were surrounded by, and to a less extent mixed with, phagocytic reticulum-cells. Admittedly, we were never able to detect any differences between the structure of the nuclei of the two types of cells, but we were inclined to attribute this failure to the extreme immaturity of the elements. We now think that most of the cells were of the same type, namely, neuroblastoma cells, and that some contained debris in their cytoplasm, but it was impossible to determine whether this was the result of phagocytosis or of cell degeneration. Either view explains Landolt's obser-

vation that cells containing debris were more numerous after deep *x*-ray therapy, although he considered that the debris had been ingested by elements which he regarded as being reticulum cells. Our photomicrographs clearly show that there are no morphological differences between the obvious tumour-cells and most of the debris-containing elements. There are, nevertheless, some cells which contain red corpuscles and polymorphs, and are thus obviously phagocytic; but whether they are tumour-cells or reticulum-cells cannot be determined.

The whole subject of the reactive proliferations of reticulum cells as found in marrow films has been discussed in detail by Nordenson (1938), who records the presence of large numbers of these elements in the vicinity of carcinomatous metastases in the marrow. It would seem, therefore, that such an appearance has not quite the pathognomonic significance that is suggested by Landolt. On the other hand, too little is known about the histological appearances of neuroblastomatous metastases in the marrow. It is, of course, possible that they are surrounded by proliferated reticulum cells, which are not sufficiently loosened from their normal attachments to be found in large numbers in material obtained by marrow puncture and suction, for as de Weerdt emphasized, " les cellules réticulo-endothéliales fixes sont exceptionelles dans les produits de ponction sternale."

There is, unfortunately, no means of differentiating, with certainty, cells which have ingested amorphous debris from those in which particulate material is present as a result of cell degeneration. In the present case the difficulty is greater than usual because no signs of phagocytosis or of cell-inclusions could be found in sections of post-mortem material. Our view is that when there is neuroblastomatous infiltration of the marrow, some tumour cells will be present in syncytial masses, while others will be more or less irregularly scattered throughout the tissue, and both may contain debris in their cytoplasm. Only if recognizable cells are present in the cytoplasm can the occurrence of phagocytosis be taken as proven.

Summary

A case of neuroblastoma of the left suprarenal gland causing Hutchison's syndrome is presented in some detail.

Radiologically the changes in the skeleton were similar to those sometimes found in the reticuloses, while sternal puncture proved to be misleading, mainly on account of the authors' ignorance of the similar cases previously recorded.

The scanty literature on the subject has been reviewed, and the nature of the so-called reticulum cells, which were found to be increased in number, has been discussed.

REFERENCES

Albertini, A. von, and Willi, H. (1938–9). *Ann. paediatr., Basel*, **152**, 129.
Fanconi, G. (1943). Ibid., **160**, 318.
Hooft, C., and Compernolle, H. (1942). *Mschr. Kinderheilk.*, **90**, 332.
Hutchison, R. (1907). *Quart. J. Med.*, **1**, 38.
Kato, K., and Wachter, H. E. (1938). *J. Pediat.*, **12**, 449.
Landolt, R. F. (1946). *Helv. paediat. Acta*, **1**, 403.
Nordenson, N. G. (1938). *Acta path. microbiol. scand.*, **15**, 362.
Patschadji, A. R. (1941). *Strahlentherapie*, **70**, 464.
Vogel, P., and Bassen, F. A. (1939). *Amer. J. Dis. Child.*, **57**, 245.
Weerdt, W. de (1939). *Rev. belge Sci. méd.*, **11**, 297, 337, 440.

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A COMPLEMENT FIXATION TEST FOR HERPES SIMPLEX INFECTIONS

BY

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The serological diagnosis of virus infections depends for the most part on neutralization and complement fixation tests. Both of these have been described in the case of herpes simplex infections, but the neutralization test is used almost exclusively at present, owing to the conflicting results obtained by complement fixation. The reports in the literature on this subject are at variance with each other. Kraus and Takaki (1925, 1926) and Takaki, Bonis, and Koref (1926) reported positive fixation with herpes virus using convalescent human and rabbit sera with a rabbit brain antigen. These workers also reported that they were able to distinguish by this method between herpes and other neurotropic viruses such as vaccinia, rabies, and the virus of Japanese B encephalitis. Their work, however, was not confirmed by Greenbaum and Harkins (1925), Tang and Castaneda (1929), and Gay and Holden (1929). Schultz and his colleagues in a series of investigations (Schultz, 1928; Schultz, Bullock, and Lawrence, 1928; Schultz and Hoyt, 1928) not only failed to demonstrate complement fixation with herpes but stated that the *in vitro* combination of antigen and antibody in the case of filterable viruses did not occur, and that the degree of fixation reported by previous workers was due, first to bacterial contamination of the virus antigens, and secondly to a lack of specificity due to inadequate controls. Bedson and Bland (1929) were able to demonstrate specific complement fixation with herpes by using an immune guinea-pig serum and an antigen prepared from guinea-pig pads. This method eliminated the non-specific and anticomplementary results produced by rabbit brain antigen and rabbit serum. Brain (1932) using the same method as Bedson and Bland was able to show that there was a definite relationship between the presence of complement-fixing antibodies and neutralizing antibodies in human sera. Myers and Chapman (1937) found no satisfactory evidence of complement fixation between several different herpes antigens and immune herpes sera, in marked contrast to the results they obtained with vaccinia and virus III. Their results were either completely negative, non-specific, or showed fixation only at a low titre. The discrepancy in these findings was probably due to several factors; first, the lack of a sufficiently

* This work, started in London, was completed on a visit to the Eliza Hall Institute, the Royal Melbourne Hospital, in October, 1949.

potent antigen; secondly, the use of reagents which tend to give non-specific or anticomplementary results; and thirdly, the lack of sufficiently critical conditions for the test. Casals and Palacios (1941) and Casals (1947) showed that some of the non-specific results with brain antigens could be eliminated by purification of the antigen by some physical method, such as by ultra-centrifugation or by freezing and thawing; by the use of homologous tissue for the production of immune sera, and by inactivation of sera at various temperatures between 56° C. and 65° C., depending upon the species from which they were derived.

The present investigation was carried out to determine whether a more satisfactory antigen could be prepared from the developing chick embryo, as has been the case with other viruses such as influenza, mumps, vaccinia, psittacosis, and more recently with toxoplasma. Preliminary experiments were carried out with antigens prepared from rabbit and mouse brain, guinea-pig pad, and the chorio-allantoic membrane. This report deals with the preparation of the chorio-allantoic membrane antigen and its use in the diagnosis of herpes simplex infections. Since this investigation was completed Hayward (1949) has published an account of the preparation and use of an antigen, in many aspects similar to the one described below and which gives similar results in the diagnosis of herpes simplex infections.

Methods and Materials

In the preliminary stages of this work an attempt was made to find the best source of antigen. Chick embryos of varying ages were inoculated by the following routes: (*a*) 9-day-old, via the yolk sac, the amniotic fluid being harvested (Nagler, 1946); (*b*) 11-day-old, via allantoic route; (*c*) 12-day-old, via chorio-allantoic route; (*d*) 13-day-old, via amniotic route. The inoculum was a suspension of a herpes-infected chorio-allantoic membrane diluted 1 in 1,000 in 10% rabbit-serum saline. After 48 hours' incubation at 36° C. fluids or membranes were harvested and tested for the infectivity titre by the pock-counting method (Burnet and Lush, 1939). It was found that these fluids or membrane suspensions containing virus, as determined by the infectivity titre, also fixed complement when tested with a positive herpes serum (human). There was little difference in the antigen titre between them, the average titre being 1/8 in the case of the amniotic and allantoic fluids, and 1/16 in the case of the chorio-allantoic membrane suspension. Similar results have recently been obtained by Hayward (1949). Occasionally, a few individual amniotic and allantoic fluids were anticomplementary. This was not noticed with the chorio-allantoic membrane suspensions, and as the latter gave such constant fixation at this titre and was easy to prepare, it was used throughout the remainder of the investigation in preference to the other methods.

Strains.—Two strains were used: (*a*) A local strain, H.S.C./Dew, isolated on the chorio-allantoic membrane from a case of acute aphthous stomatitis in this hospital, and since maintained by passage in the chick embryo; (*b*) strain H.F.E., the standard egg-adapted strain used at the Walter and Eliza Hall Institute, the Royal Melbourne Hospital. Both strains behaved in the same way on animal inoculation, although the H.F.E. strain had a higher infectivity titre. Owing to the close antigenic relationship between all herpes simplex strains, no distinction between these two strains is made in this paper.

Diluting Fluid.—Buffered calcium magnesium saline *p*H 7.2 (Mayer, Osler, Bier, and Heidelberger, 1946) was used as a diluting fluid throughout, in preference to physiological saline.

Antigens.—Twelve-day-old chick embryos were inoculated by the chorio-allantoic

route with a suspension of herpes-infected chorio-allantoic membrane so diluted in 10% rabbit serum saline as to give confluent lesions (approximately 1/1,000 in this laboratory). After 48 hours' incubation at 36° C. embryos were placed in the ice-box for one hour and the membranes were then removed. Those showing confluent lesions were pooled, washed in sterile saline, and ground into a fine emulsion. Sufficient diluting fluid was added to make a 20% suspension. The suspension was then alternately frozen and thawed six times in a mixture of CO_2 and alcohol. This process did not appear to increase the antigen titre but helped to sediment by flocculation extraneous protein material. After centrifugation at 2,500 r.p.m. in an angle centrifuge for one hour the supernatant was removed. This constituted the herpes antigen (herpes-C.A.M. antigen*). Membranes from normal embryos (14-day) were harvested and prepared in exactly the same way. This constituted the normal or control antigen (normal-C.A.M. antigen). Both antigens were stored at −20° C. Merthiolate (1 in 10,000) was added as preservative.

Sera and Antisera.—Acute-phase (one to four days) and convalescent-phase (seven to 21 days) sera were collected from all cases of herpes simplex infection under investigation, and also from rabbits and guinea-pigs (used for virus isolation) before inoculation and again 21 days later. A hyper-immune guinea-pig (P/12) was prepared by a series of injections with a formolized guinea-pig pad suspension, followed by injections with living virus. All sera were stored at −20° C. without preservative.

The temperature at which sera were inactivated before use in this test was found to be of considerable importance in eliminating non-specific results. Casals and Palacios (1941) have shown that the non-specific reaction in normal serum could be destroyed by heat without materially affecting the specific antibody titre, the temperature of inactivation depending upon the animal species. In this case it was found that guinea-pig sera and most human sera could be successfully inactivated at 56° C. for 20 minutes, but nearly all rabbit sera and a few human sera produced non-specific fixation even after inactivation at this temperature. Two positive sera (human and rabbit) diluted 1 in 2 in buffer were inactivated at temperatures ranging from 56° C. to 66° C. for 15 minutes. The original serum titre of 1 in 16 in both cases showed no fall in titre at 62° C.; at 64° C. the titre fell to 1 in 8 and at 66° C. to 1 in 4. Guinea-pig sera tested in the same way showed a slight reduction in titre after inactivation at 60° C. and were not therefore inactivated beyond this temperature. All other sera were therefore inactivated at 62° C. for 15 minutes.

Haemolytic System.—Defibrinated sheep cells (formalized) were washed three times in saline, made up to 3% in the diluting fluid, and mixed with an equal quantity of haemolysin diluted to contain 5 M.H.D. Cells were sensitized for 30 minutes at 37° C.

Neutralization Tests.—To obtain some correlation between the presence of complement-fixing and the neutralizing antibody, sera were tested by both methods. The neutralization test was carried out by the method suggested by Burnet and Lush (1939). In this test use is made of the fact that the virus of herpes simplex produces visible lesions or pocks after inoculation on to the chorio-allantoic membrane. By inoculating mixtures of serum and virus in constant proportions on to the chorio-allantoic membrane, it can be shown whether a particular serum will inhibit the production of such lesions or not. In practice, individual pock-counts should be reduced by at least 50% before it can be stated that a particular serum contains neutralizing antibodies. In this investigation the results were quite clear-cut. Sera with neutralizing antibody present produced at least a 90% reduction in the pock-count in all cases.

* C.A.M. is used as an abbreviation for chorio-allantoic membrane to designate the source of the antigen.

Complement Fixation Test

Antigen Titration.—The titres of the herpes-C.A.M. and normal-C.A.M. antigens were determined by the method set out below using titration with both a normal and a positive serum (Table I). Both these sera (P/12) were tested for the presence of

TABLE I

COMPLEMENT-FIXATION TITRES OF HERPES-C.A.M. AND NORMAL-C.A.M. ANTIGENS

Sera*	Antigen Dilutions in Ca++ Mg++ Saline											
	Herpes-C.A.M. Antigen						Normal-C.A.M. Antigen					
	1:2	1:4	1:8	1:16	1:32	Antigen Control	1:2	1:4	1:8	1:16	Antigen Control	Serum Controls
P/12 acute N.A.(−) ..	0	0	0	0	0	0	0	0	0	0	0	0
P/12 hyper-immune N.A. (+)	4	4	4	3	1		0	0	0	0		0

4 = complete fixation; 3, 2, 1 = partial fixation; 0 = no fixation; 2 = end-point.
N.A. = Neutralizing antibody present (+) or absent (−).

* Both sera were used diluted 1 in 2 and would therefore contain excess antibody if present.

neutralizing antibody, as were all other sera used in this investigation. They were used as control sera throughout these tests but are not included in all the tables.

Preliminary Titration.—Before testing a batch of sera a preliminary titration of the complement was carried out in the presence of the antigen and a negative serum. The antigen was used diluted, as in the test proper, to contain 8 units per unit volume (as titrated above). The serum was used diluted 1 in 2. After fixation for one hour at 37° C. the sensitized cells were added. The dilution of complement, which in the presence of antigen and the negative serum showed 50% haemolysis (++), was taken as the end-point; this end-point was determined entirely by the appearance of partial fixation based on experience. It was found, after repeated tests, that this short period of fixation gave good results and that the amount of complement necessary for the test could be assessed more accurately by this preliminary titration. The antigens were never found to be anticomplementary by themselves, but there was usually a slight reduction in titre in the tube containing complement, antigen, and serum. The complement was used diluted to 2 M.H.D. throughout the test. Although the complement unit was based on the short fixation period, the actual amount of complement in the test proper after fixation overnight at 4° C. was likely to be slightly less than 2 units.

Test Proper.—Unit volumes of 0.1 ml. were used throughout the test. Sera were inactivated as described above and serial twofold dilutions made from 1 : 2 to 1 : 128. Antigen (8 units) was added to each tube, followed by 2 M.H.D. complement. Each test was set up with the herpes-C.A.M. and normal-C.A.M. antigens and with a positive and negative serum. After overnight fixation at 4° C. the sensitized cells were added (0.2 ml.). The results were read after a further period of incubation at 37° C. for 30 minutes, the dilution of serum showing 50% fixation being taken as the end-point.

Results of Complement Fixation

The results of this test in various types of herpes simplex infection are shown in the following tables.

Virus Identification.—This test was used as a means of rapid identification of

lesions on the chorio-allantoic membrane. Suspensions of several such membranes were made and tested by the method described above, using a normal and a vaccinia-infected membrane as controls. The results of these tests showed specific fixation to a titre of 1 in 16 with the membrane under test and a positive herpes serum, and no fixation with the control membranes.

In addition, the antigen was centrifuged at 15,000 r.p.m. for one hour in a horizontal centrifuge in order to sediment the virus elementary bodies. The supernatant was removed and the deposit resuspended in a volume of saline equivalent to the original uncentrifuged material. The complement-fixation titre of the supernatant showed a twofold drop in titre and over a 1,000-fold drop in the infectivity titre. The resuspended elementary particles fixed complement, though to a lesser extent than the original material, but the infectivity titre was the same. This suggested the presence of a soluble antigen in the supernatant. Since this work was completed Hayward (1949) has clearly shown that the complement-fixing antigen is due to a specific soluble substance which can be separated from the virus particle by high-speed centrifugation.

Complement Fixation Titres of Acute and Convalescent Sera.—The titres of four sera are shown in Table II. The sera P/2 were obtained from a guinea-pig

TABLE II

COMPLEMENT-FIXATION TITRES OF ACUTE AND CONVALESCENT-PHASE SERA WITH HERPES-C.A.M. AND NORMAL-C.A.M. ANTIGENS

Sera	N.A.	Serum Dilutions in Ca^{++} Mg^{++} Saline											
		Herpes-C.A.M. Antigen						Normal-C.A.M. Antigen					
		1:2	1:4	1:8	1:16	1:32	Antigen Control	1:2	1:4	1:8	1:16	Antigen Control	Serum Controls
P/2 (Ac)	−	0	0	0	0	0	0	0	0	0	0	0	0
P/2 (Con)	+	4	4	4	2	0		0	0	0	0		0
R/1 (Ac)	−	0	0	0	0	0		0	0	0	0		0
R/1 (Con)	+	4	4	4	3	1		0	0	0	0		0
P/12 + control	+	4	4	4	3	1		0	0	0	0		0

N.A. = Neutralizing antibody. See Table I for key to other abbreviations.

inoculated intracutaneously with vesicle fluid from a case of Kaposi's varicelliform eruption. Sera R/1 were obtained from a rabbit inoculated by the corneal route with mouth washings from a case of acute aphthous stomatitis. The acute-phase sera were collected before inoculation.

Complement Fixation in Primary Herpes Infection.—The serum titres in various forms of primary herpes infections are shown in Table III.

These figures demonstrate the characteristic rise in antibody which occurs as a result of the primary infection with herpes. This is in accord with the findings established by the neutralization test. The serum titres in one case of recurrent Kaposi's varicelliform eruption in an adult (Boake and Dudgeon, 1950) are in marked contrast to those found in the infantile type. Antibody was present in the early

TABLE III

COMPARISON BETWEEN THE COMPLEMENT-FIXATION TITRE AND PRESENCE OF NEUTRALIZING ANTIBODY

Sera	Acute-phase Serum		Convalescent-phase Serum	
	Complement Fixation Titre	Neutralizing Antibody	Complement Fixation Titre	Neutralizing Antibody
(a) Acute Aphthous Stomatitis of Infants				
Case 1*	0	−	8	+
,, 2*	0	−	16	+
,, 3*	0	−	16	+
,, 4*	0	−	8	+
,, 5*	0	−	16	+
,, 6	0	−	16	+
(b) Kaposi's Varicelliform Eruption				
Primary:				
1†	0	−	16	+
2†	0	−	16	+
3†	0	−	8	+
4†	0	−	16	+
Recurrent:				
5 (adult)†	16‡	+	16	+
(c) Recurrent Herpes in Adults				
Case 1	8	+	8	+
,, 2	16	+	16	+
,, 3	16	+	16	+
,, 4	16	+	16	+
,, 5	8	+	8	+

* Virus isolated from mouth washings. † Virus isolated from cutaneous lesions. ‡ Serum taken on fourth day of disease.

stages of the infection and no increase in antibody was detected, as was the case with other forms of recurrent herpes infection (Table IIIc).

The antibody titre amongst the normal sera (Table IV) shows a well-distributed scatter amongst the positive sera and complete absence of antibody in the negative sera. Similar findings with normal human sera have been reported recently by

TABLE IV

THE ANTIBODY LEVEL IN NORMAL HUMAN SERA

Type of Serum	Number of Sera showing Fixation Serum Antibody Titre				
	1:2	1:4	1:8	1:16	1:32
Positive:					
30 neutralizing sera	2	7	12	8	1
Negative:					
10 neutralizing sera	0	0	0	0	0

Hayward (1950). This is in accord with the "all or none" phenomenon of herpes antibody (Burnet, 1945), which persists at a fixed level after the primary infection. No fluctuations in antibody titre can be detected by the neutralization test and the

presence of antibody does not appear to influence, or to be influenced by, recurrent attacks of herpes which are so common. It was amongst this group that several non-specific results were obtained before inactivation of the sera at 62° C.

Discussion

A complement-fixation test for the virus of herpes simplex, using an antigen prepared from the chorio-allantoic membrane of the chick-embryo, has been described. With few exceptions, antigens prepared from the chick-embryo have several advantages over antigens prepared from animal and, in particular, brain tissue. They are easy to prepare, they have a high virus content, are bacteriologically sterile, and the risk of non-specific results is reduced. At the same time control antigens from uninfected tissue are easily prepared. In this particular case the chorio-allantoic membrane was used as the source of antigen in preference to the amniotic and allantoic fluids which, although they showed positive fixation, were seldom as constant in titre and on occasions were anticomplementary. The interpretation of any complement-fixation reaction depends to a great extent on the specificity of the reaction. The more sensitive and more specific the test can be made, the greater is its value. This quality of specificity is largely dependent on the antigen itself. Kraus and Takaki (1925) and Takaki, Bonis, and Koref (1926) used an antigen prepared by boiling the supernatant from a rabbit-brain emulsion. Such treatment would almost certainly greatly reduce the antigen titre. Hayward (1949) has shown that the antigen titre is markedly reduced after heating to 56° C. for 20 minutes. Bedson and Bland (1929) prepared an antigen of high virus content and used a prolonged period of fixation (overnight at 4° C.) for their tests. The results they obtained were specific. The reaction that may occur between certain tissue antigens and normal sera in some cases fixes complement (Casals and Palacios, 1941). The substance present in the antigen responsible for this reaction can be sedimented at 20,000 r.p.m. (Kidd and Friedewald, 1942) and the antibody-like substance in the serum can be destroyed by heat (Casals and Palacios, 1941 ; Mackie and Finkelstein, 1928). At the same time antisera should be prepared by inoculation of animals with homologous tissue to prevent the formation of antibody to that particular tissue. In one instance in this investigation a rabbit serum was used as a control serum. It fixed complement with both the herpes and normal antigen to a titre of over 1/256. It was later discovered that this animal had three months previously received a course of injections of allantoic fluid containing influenza virus. This was presumably the result of the formation of antibody to the chick-embryo tissue. In the present investigation non-specific results were almost entirely eliminated by (1) the use of the chorio-allantoic membrane antigen ; (2) the preparation of hyper-immune sera with homologous tissue ; (3) inactivation of the sera at 62° C. for 15 minutes. The temperature of inactivation is obviously of considerable importance. The fact that the specific herpes antibody titre in human and rabbit sera was not altered after inactivation at 62° C. for 15 minutes offers an alternative method of elimination of non-specific fixation. This has been noted in connexion with sera from other virus infections. The antigens were not regularly centrifuged at high speed as the results obtained without this process were satisfactory. On the few occasions that such antigens were centrifuged at 15,000 r.p.m. for one hour they showed only a small drop in antigen titre ($\times 2$) but a considerable drop in infectivity

titre ($\times$ 1,000), indicating the presence of a soluble antigen. This confirms the findings recently reported by Hayward (1949). The results of this test in the various forms of herpetic infection are in complete accord with the findings made by the neutralization test. The presence of serum neutralizing antibody as shown in this series was paralleled by the presence of complement-fixing antibody, though the two are not necessarily identical. More work will be needed to determine the relationship between them. In the few cases of recurrent herpes infection investigated there did not appear to be any increase in antibody after the acute phase, and many of the patients had a high titre of antibody, as determined by this method, at the time of infection. The case of recurrent Kaposi's varicelliform eruption was an example of this particular point. In this case the serum was examined on the fourth day (acute-phase) and again on the twenty-first day; no rise in titre was detected. It is possible, of course, that with a more potent antigen changes in antibody titre might be detected, but with this particular method the antibody titre did not appear to change, remaining constant throughout an exacerbation. Several cases of primary herpes infection were investigated and all showed the characteristic rise in antibody which occurs as a result of the infection, in marked contrast to all the recurrent forms of the condition investigated. Complement fixation was also used as a means of virus identification in the laboratory. Lesions on the chorio-allantoic membrane may sometimes be difficult to interpret, as in one instance recently reported by Kipping and Downie (1947). By this method the distinction between variola, vaccinia, and herpes can be made more readily should the occasion arise.

The test appeared to be of value as an alternative diagnostic method in herpes simplex infections, in particular in those instances where a large number of sera have to be examined. It may also be useful in following the course of antibody production in cases of recurrent herpes infections, but so far in the few cases examined no change in antibody has been detected.

Summary

A complement fixation test for the virus of herpes simplex is described.

The preparation of the antigen and the use of the test in various types of herpes simplex infections are described.

Complement fixation as a means of virus identification is also described.

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REFERENCES

Bedson, S. P., and Bland, J. O. W. (1929). *Brit. J. exp. Path.*, **10**, 393.
Boake, W. E., and Dudgeon, J. A. To be published.
Brain, R. T. (1932). *Brit. J. exp. Path.*, **13**, 166.
Burnet, F. M. (1945). *Virus as Organism*, p. 49. Harvard Univ. Press.
—— and Lush, D. (1939). *Lancet*, **1**, 629.
Casals, J. (1947). *J. Immunol.*, **56**, 337.
—— and Palacios, R. (1941). *J. exp. Med.*, **74**, 409.

Gay, F. P., and Holden, M. (1929). *J. infect. Dis.*, **45**, 415.
Greenbaum, S. S., and Harkins, M. J. (1925). *Arch. Derm. Syph., Chicago*, **11**, 789.
Hayward, M. E. (1949). *Brit. J. exp. Path.*, **30**, 520.
—— (1950). *Lancet*, **1**, 856
Kidd, J. G., and Friedewald, W. F. (1942). *J. exp. Med.*, **76**, 543.
Kipping, R. H., and Downie, A. W. (1948). *Brit. med. J.*, **1**, 247.
Kraus, R., and Takaki, J. (1925). *Med. Klinik.*, **21**, 1872.
—— —— (1926). *Klin. Wschr.*, **39**, 624.
Mackie, T. J., and Finkelstein, M. H. (1928). *J. Hyg., Camb.*, **28**, 172.
Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M. (1946). *J. exp. Med.*, **84**, 535.
Myers, R. M., and Chapman, M. J. (1937). *Amer. J. Hyg.*, **25**, 16.
Nagler, F. P. O. (1946). *Aust. J. exp. Biol. med. Sci.*, **24**, 103.
Schultz, E. W. (1928). *J. Immunol.*, **15**, 229.
—— Bullock, L. T., and Lawrence, F. (1928). Ibid., **15**, 243.
—— and Hoyt, J. (1928). Ibid., **15**, 411.
Takaki, J., Bonis, A., and Koref, O. (1926). *Z. ImmunForsch.*, **47**, 431.
Tang, F. F., and Castaneda, M. R. (1929). *J. Immunol.*, **16**, 151.

J. clin. Path. (1950), **3**, 248.

DETERMINATION OF BILIRUBIN WITH PRECIPITATION OF THE PLASMA PROTEINS

BY

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Methods for the estimation of bilirubin are of two general types: those in which the bilirubin is caused to react with diazotized sulphanilic acid through the agency of added ethanol, which causes precipitation of the plasma proteins; and those in which some other agent is used to bring the bilirubin into reaction, without precipitating the proteins. The former methods (for example, those of van den Bergh, 1918, quoted by Thannhauser and Andersen, 1921; Jendrassik and Czike, 1928; Haslewood and King, 1937) all suffer from the disadvantage that some bilirubin may be lost in the protein precipitate. Van den Bergh recognized the difficulty, and Haslewood and King measured the loss to be about 10% in the case of highly icteric sera, but pointed out the impossibility of estimating what it might be with sera containing only small amounts of the pigment. Methods not entailing precipitation of the plasma proteins are most prominently represented by the procedures of Malloy and Evelyn (1937), Rappaport and Eichhorn (1943), and Gray and Whidborne's (1946) modification of the latter. Gray and Whidborne found an average difference of 40% between a "precipitation" method on the one hand and two "non-precipitation" methods on the other, when these were applied to sera and plasma from cases of jaundice. Because we have been dissatisfied with the performance of the "non-precipitation" methods thus far described, for example, on account of the very pale colours produced in the Malloy and Evelyn method, which employs a very high dilution (1 in 25), and the frequently "impure" colours produced by the Rappaport and Eichhorn method, we have sought another procedure.

After trying several polyhydric alcohols (glycerol and glycols), whose hydroxyl groups were counted on to favour the retention of the plasma proteins in solution, a mixture of glycerol (35), ethanol (25), and water (25) was adopted. This furnished a favourable medium for the diazotization of bilirubin, at a 1 in 10 dilution so that the colours to be measured were reasonably intense; and there was no tendency of the plasma proteins to precipitate. The colours obtained were indistinguishable from those produced from pure bilirubin in ethanol, and the procedure devised was quick and easy. Recovery of added bilirubin averaged nearly 100%; and there was good agreement between values obtained by this "glycerol method" and the method of Malloy and Evelyn. We reported the procedure and results to the Biochemical Society (King and Coxon, 1949), but later discovered that with certain blood sera (from the cord blood of jaundiced babies) erroneously low results were obtained. These sera were highly "indirect," in the sense that the van den Bergh reaction was

very slow to develop, and the colour continued to increase for a very long time. Likewise in the "glycerol method" the bilirubin reacted slowly; the colours frequently took several hours to reach their maximum, and in several instances never attained a sufficient intensity to represent all the bilirubin known to be present. With the Malloy and Evelyn method (methanol) the colour development also seemed to be retarded somewhat, but not nearly to the same extent as it was with glycerol.

Only with ethanol in adequate concentration does the diazo reaction appear to go to completion in a reasonable time (<30 minutes); but it is necessary to use a higher dilution of the serum and reagents in ethanol (i.e., 1 in 10 instead of the previous 1 in 4 dilution of Haslewood and King, 1937), and to allow for the bilirubin azo dye distributed through the precipitated protein, in order to obtain colours which represent all the bilirubin present. With highly icteric sera it is necessary also to dilute the sera with a buffer in order to diminish the concentration of bilirubin sufficiently to ensure that all of it will react with the diazo reagent.

The method to be described yields colours which are spectrally identical with those produced under similar conditions from pure bilirubin, gives reasonably accurate recovery of added pure bilirubin, and compares favourably in the results procured on a wide range of icteric sera with the Malloy and Evelyn procedure. The colours are equally suitable for Duboscq and photoelectric colorimeters.

Reagents

Diazo A.—Sulphanilic acid (1 g.) dissolved in 1 l. of 0.25 N-HCl.

Diazo B.—$NaNO_2$ 0.5 g. in 100 ml. water.

Protein Precipitant.—Saturated $(NH_4)_2SO_4$ solution.

Diluent.—Ethanol 85%.

Phosphate Buffer Solution.—$Na_2HPO_4.12H_2O$ 3.6 g. in 100 ml. water.

Artificial Methyl Red Standard (identical in colour with diazotized bilirubin).—2.9 mg./l. in acetate buffer at *p*H 4.63. This is made in two stages. A stock solution is prepared containing 0.29 g. of pure methyl red (*o*-carboxy benzene azodimethylaniline) in 100 ml. of glacial acetic acid; 1 ml. of this stock solution is placed in a litre flask together with 5 ml. of glacial acetic acid. About 500 ml. of water are added,

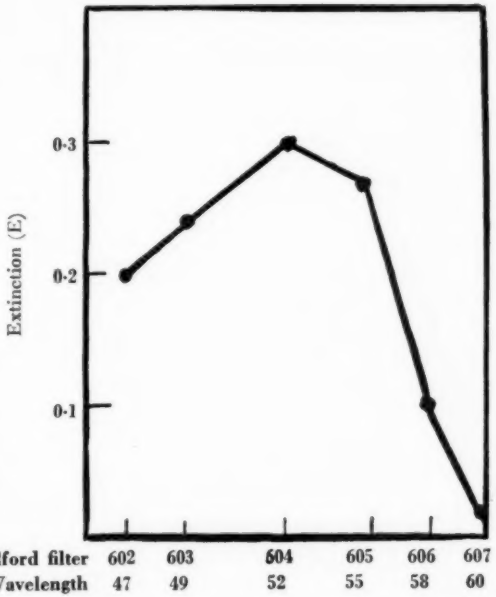

FIG. 1.—Spectral absorption curve of the artificial methyl red standard used for comparison with standard bilirubin azo-dye solutions and tests prepared from blood sera with diazotized sulphanilic acid. (Spekker absorptiometer; Ilford spectrum filters; wavelength cμ.)

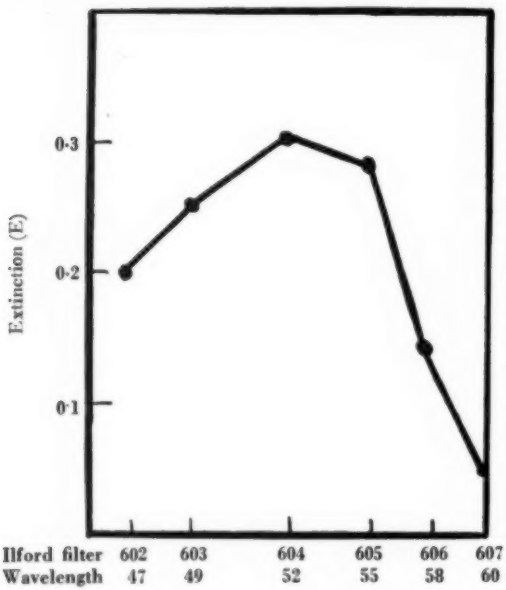

FIG. 2.—Spectral absorption curve of standard bilirubin azo-dye solution used. Final concentration corresponding to 0.4 mg. bilirubin/100 ml. of coloured solution; equivalent to 4 mg. bilirubin/100 ml. serum at a 1 in 10 dilution.

followed by 14.4 g. of crystalline sodium acetate. After the salt has dissolved, further water is added to make up the volume to 1 l. Its spectral curve is given in Fig. 1, and that of a pure bilirubin-azo-dye standard in Fig. 2.

This is the same standard as was used by Haslewood and King (1937). In that method it represented 1.6 mg. of bilirubin/100 ml. of plasma, with the plasma at a final dilution, after diazotization and treatment with ethanol, etc., of 1 in 4. In the present method, with a final dilution after diazotization of 1 in 10, the standard represents 4 mg. of bilirubin/100 ml.

Standard Bilirubin Solution.—For testing the accuracy of the method a standard solution of bilirubin was made of 10 mg. "pure" bilirubin (East Kodak) dissolved in 100 ml. of chloroform. It was found necessary to heat the mixture gently under reflux to bring the bilirubin into solution in the chloroform.

Procedure

The volumes suggested below provide 10 ml. of test solution (8 ml. after filtration), which is a convenient quantity for the cells of most photoelectric and Duboscq colorimeters. Smaller volumes in the same proportion may be taken, depending on the capacity of the cups of the colorimeter employed; as little as 0.1 ml. of serum has been successfully used.

Take 1 ml. of plasma or serum in a test tube. Add 0.5 ml. of diazo reagent (when the type of van den Bergh reaction, e.g., "direct," may be noted).* Add 0.5 ml. of $(NH_4)_2SO_4$ solution and make up to 10 ml. with diluent (85% ethanol). Mix well and stopper, and leave the solution lying horizontally with an even distribution of the precipitate for 30 minutes. Filter and then measure the extinction (optical density) of the test in a photoelectric colorimeter (or Duboscq colorimeter with a 0.5 D grey screen, Haslewood and King, 1937), using a green light filter, and making the zero setting with water. Measure the extinction of the standard with the zero setting made with water. Direct comparison of the test with the standard solution may also be made in any type of visual colorimeter.

* An estimation of the "direct" bilirubin may be made by diluting this mixture of 1 ml. serum and 0.5 ml. diazo reagent to 10 ml. with water, and reading in the colorimeter at, e.g., 1 minute ("1 minute direct"), or at various intervals to follow the course of development of the colour. Compare the direct-indirect quotient (D.I.Q.) of Grey and Whidborne (1947).

Calculation.—Calculations are made as follows:

Photoelectric Instruments.—When E_T = the extinction of the azobilirubin in the test, E_S = the extinction of the standard, and 4 = the equivalent strength of the artificial methyl red standard in terms of mg. bilirubin in 100 ml. of plasma:

$$\text{mg. bilirubin/100 ml. plasma} = \frac{E_T}{E_S} \times 4.$$

Duboscq Colorimeters.—When T = the reading in mm. of the test, S = the reading of the standard, and 4 = the equivalent strength of the standard:

$$\text{mg. bilirubin/100 ml. of plasma} = \frac{S}{T} \times 4.$$

Additional Procedure for Sera of High Bilirubin Content.—If the value of E_T is more than twice that of E_S (or, as mg. of bilirubin, is greater than 8), a suitable dilution of the original serum with the phosphate buffer solution may be made (e.g., 1 in 3, 1 in 10), and the procedure repeated. Since this additional step involves a dilution of the serum, (e.g., by 1 in 3), the resultant reading must, of course, be multiplied by the dilution factor to give the true concentration. Sera which are obviously icteric may be diluted in the first instance.

Results

Recoveries.—The recoveries achieved by this method, when tested on sera to which known amounts of bilirubin had been added, are shown in Table I.

TABLE I

RECOVERY OF BILIRUBIN ADDED TO SERA

Serum Bilirubin (mg./100 ml.)	Bilirubin Added to Serum (mg./100 ml.)	Total Bilirubin Found (mg./100 ml.)	Added Bilirubin Recovered (mg./100 ml.)	Recovery (%)
Normal sera				
0.46	1.6	1.96	1.5	94
0.41	1.6	1.91	1.5	94
0.23	4.65	4.55	4.32	93
0.23	4.60	4.65	4.42	96
0.42	9.3	9.3	8.9	95
0.42	9.7	9.4	9.0	93
Undiluted sera of haemolytic jaundice				
8.0	1.8	9.7	1.7	94
7.9	1.8	9.7	1.8	100
7.6	4.5	11.5	3.9	87
7.4	4.5	10.9	3.5	78
Haemolytic jaundice serum diluted 1 *to* 5 *with buffer* (results multiplied by 5)				
7.7	8.4	15.9	8.2	98
7.5	7.8	15.5	8.0	102
7.6	22.8	30.7	23.1	101
7.2	22.6	29.8	22.6	100
Obstructive jaundice serum diluted 1 *to* 5 *with buffer* (results multiplied by 5)				
9.55	8.65	17.7	8.15	94
9.55	8.4	18.2	8.65	102
17.4	21.8	39.1	21.7	100
17.6	21.8	39.6	22.0	101

The technique by which these figures were obtained was as follows:

A solution was prepared to contain 10 mg. of bilirubin in 100 ml. of mixed ethanol and chloroform by evaporating 20 ml. of the bilirubin solution in chloroform on a water bath down to about 5 ml. and then making it up again to 20 ml. with absolute ethanol.

Various volumes of this ethanol-chloroform solution of bilirubin were then incorporated in mixtures of serum and diluent and then diazotized. A second set of mixtures was made up at the same time substituting phosphate buffer for serum. Each test solution was then read, and the recovery calculated by comparing the amount added (as indicated by the bilirubin + phosphate-buffer mixture) with the amount found in the mixture containing serum, both being referred to the artificial standard. (This method of estimating recoveries obviates any inaccuracy arising from impurity of the bilirubin used and from any deterioration of the standard solution of bilirubin, its behaviour in the phosphate-containing mixture having been established in the first instance when it was freshly made up.) The straight line relationship between the amounts of bilirubin added in standard solutions and the colour development is illustrated in Fig. 3.

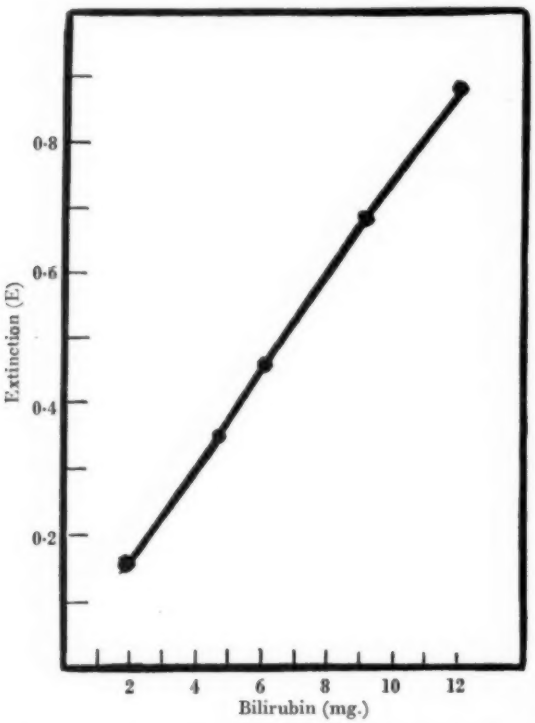

FIG. 3.—Graph of relation between colour density and concentration of bilirubin. Abscissae: mg. of bilirubin equivalent per 100 ml. of serum; actual bilirubin present in the bilirubin azo-dye solution, 0.1 of that shown. (Spekker absorptiometer; Ilford 604 spectrum green filter.)

Relation of Colour Development to *p*H.—Using a green filter (Ilford spectrum green 604 or 624), the photoelectric reading varies by approximately 5% over the range *p*H 2.9 to 3.5. To assess the relevance of this variation to the estimation of bilirubin in plasma and serum, specimens of these fluids were pooled in batches, and samples from each batch were then put through the estimation procedure and the resulting mixtures tested by the glass electrode. All gave *p*H values within the range 3.1 to 3.4. It may thus be inferred that the maximum error to be expected in an estimation from this source will not exceed 5%.

Time Course of Diazo Reaction.—Table II gives a series of readings on the same specimen of serum at varying intervals from the time of carrying out the diazotization

TABLE II

COLOUR (MG. BILIRUBIN/100 ML. SERUM) OF BILIRUBIN AZO-DYE TEST SOLUTION FROM "HIGHLY INDIRECT" SERUM

Filtered at	Read in Photoelectric Colorimeter at					
	2 min.	30 min.	1 hr.	3 hr.	5 hr.	24 hr.
5 min.	16.3	15.1	15.1	—	15.1	15.0
30 min.	—	18.0	18.0	18.3	17.7	18.9*
1 hr.			18.0	17.7	18.0	19.2*
3 hr.				17.4	17.2	17.7
5 hr.					17.7	18.9*
24 hr.						17.9

* Slightly cloudy. Eel photoelectric colorimeter and Ilford 624 bright spectrum green filter were used.

and addition of the diluent to the time of filtration. The results (which are typical of several obtained from sera giving an indirect van den Bergh reaction) show that a reading made after the reaction has been allowed to proceed for 30 minutes will give a reliable estimate of the bilirubin level, since the curve becomes horizontal beyond that point on the time scale. This conclusion was confirmed by a comparison (Table III) of the values obtained of using the present procedure and the Malloy and Evelyn (1937) method of estimation.

Necessity for Additional Dilution of Highly Icteric Samples.—It has been reported by Gray and Whidborne (1946) that proportionality between colour density and concentration of azobilirubin ceases to hold good at high concentrations. With any method of estimation, where a natural or artificial standard is employed, it is essential, in keeping with the general principles governing colorimetric procedures, to ensure

TABLE III

BILIRUBIN ESTIMATIONS IN MISCELLANEOUS "DIRECT" AND "INDIRECT" SERA

Present Method (Modified Haslewood-King) (mg. Bilirubin/100 ml.)	Malloy-Evelyn Method (mg. Bilirubin/100 ml.)
0.2	0.5
1.0	1.0
1.6	1.3
1.6	1.5
1.6	1.4
1.9	1.8
2.3	2.0
2.6	3.0
3.4	3.3
3.5	3.3
3.7	3.5
4.1	3.2
5.0	4.9
5.3	4.7
8.0	9.9
8.9	8.2
12.7	16.6
16.0	16.4
26.6	26.0
Average: 5.8	5.9

Root mean square of the differences: 1.1 mg.

TABLE IV

BILIRUBIN ESTIMATIONS ON SERA GIVING STRONG "DIRECT" REACTION

Present Method (Modified Haslewood-King) (mg. Bilirubin/100 ml.)	Malloy-Evelyn (mg. Bilirubin/100 ml.)
Obstructive jaundice sera	
5.5	6.0
7.5	9.2
9.0	11.3
9.4	11.3
13.0	16.6
14.0	17.0
14.6	16.2
15.5	19.6
Average: 11.1	13.4
Root mean square of the differences: 2.6 mg.	
"*Artificial direct*" *sera :* (*human bile added to normal sera*)	
1.7	0.9
2.2	2.8
2.6	2.4
3.1	3.2
3.4	3.3
3.5	3.5
4.6	5.2
5.0	4.5
5.7	6.4
6.9	6.9
6.9	8.3
7.4	8.3
7.9	6.6
8.3	7.4
10.6	12.8
12.4	15.4
Average: 5.8	6.1
Root mean square of the differences: 0.4	

that the standard and test solution do not differ widely in concentration. Our experiments, while confirming the general conclusions of Gray and Whidborne, have demonstrated that, up to a figure which exceeds by a safe margin double that represented by the artificial standard, Beer's law is satisfactorily obeyed (Fig. 3): for this reason we have suggested this level (i.e., 8 mg. bilirubin/100 ml. plasma) as the "critical level" above which further dilution should be resorted to. This additional step can be carried out very rapidly, and with a minimum of extra trouble, by the expedient mentioned in the section dealing with the technique of the estimation. Dilution of the serum is carried out with buffer and the diluted serum treated with diazo reagent. In contrast with the experience of Gray and Whidborne, we have found evidence of incomplete coupling at high concentrations of bilirubin, and that more diazo reagent would under these conditions produce more colour. Serial dilutions as recommended here will ensure a sufficiency of the reagent for the complete conversion of all the bilirubin present into the azo dye.

The Phosphate Buffer Solution.—Some previous experiments in this laboratory (Wootton, 1946) had suggested that a 3.6% (w/v) solution of $Na_2HPO_4.12H_2O$ would, when added to Ehrlich's diazo reagent, behave as regards its buffering effect in a

TABLE V

BILIRUBIN ESTIMATIONS ON BILE DILUTED WITH BUFFER AND BLOOD SERUM*

Bile	Diluted with Buffer		Diluted with Serum	
	Present Method	Malloy-Evelyn	Present Method	Malloy-Evelyn
1	3.4	3.7	4.2	4.4
2	4.3	5.6	4.8	5.7
3	1.6	1.7	1.9	2.0
4	3.1	3.2	3.5	3.8
5	6.6	8.3	7.1	8.8
Average:	3.8	4.5	4.3	4.9

* Both mixtures gave strong " direct " van den Bergh reactions. Results expressed as mg. bilirubin/100 ml. of mixture.

TABLE VI

BILIRUBIN ESTIMATIONS ON SERA GIVING " INDIRECT " OR " DELAYED DIRECT " REACTION

Present Method (Modified Haslewood-King) (mg. Bilirubin/100 ml.)	Malloy-Evelyn (mg. Bilirubin/100 ml.)
1.1	0.8
1.8	2.1
2.0	1.6
2.2	1.7
2.9	2.6
3.2	3.4
3.2	3.3
3.3	2.5
3.7	3.0
3.7	3.7
4.0	4.1
5.0	4.2
5.3	5.0
6.1	4.0
6.5	6.4
Average: 3.6	3.2
Root mean square of the differences: 0.7 mg.	
Bile-stained fluid from peritoneum : " mixed " or " delayed direct " reaction	
(*a*) Diluted with Phosphate Buffer	
2.7	2.7
3.6	3.0
4.1	4.1
4.7	4.0
4.9	4.5
5.6	5.2
6.4	5.7
6.6	6.0
(*b*) Diluted with Serum	
3.9	3.8
5.1	4.8
5.3	5.2
6.3	6.2
Average: 4.9	4.6
Root mean square of the differences: 0.4 mg.	

manner closely resembling serum. Confirmation of this suggestion was found in the behaviour of this buffer and of serum towards varying amounts of the diazo reagent, when similar *p*H values and similar colours were obtained in test solutions containing the same amounts of bilirubin.

Comparison of Methods.—To assess the comparative performance of the procedure put forward, a series of parallel estimations has been made on some samples of serum by the present method and that of Malloy and Evelyn (1937). In order to cover a greater range of bilirubin concentrations than could be obtained from the random use of samples obtained from routine material, a number of synthetic mixtures of bile and serum were also analysed in the same way. The results of these experiments are given in Tables III, IV, V, and VI.

Discussion

The high dilution entailed in the Malloy and Evelyn method is a considerable drawback since, with colorimeter cells of the usual 1 cm. depth, the readings of serum bilirubin levels below 2.5 mg. bilirubin/100 ml. plasma are so small as to be difficult to measure at all accurately, while the deeper cells of most instruments require more than the 10 ml. of fluid that is available from 1 ml. of plasma. With Duboscq colorimeters the difficulty of reading such pale solutions is even greater.

The Rappaport and Eichhorn method gives a considerably greater depth of colour for a given bilirubin concentration. But the colours obtained are often very different from that of the standard, and, apart from the relatively complex nature of the solutions employed, there seems to be some doubt in regard to the efficiency as well as the mode of operation of their "indirect" reagent mixture. The latter contains caffeine, citrate, and urea. Jendrassik and Czike (1928), in the course of a description of the use of caffeine sodium benzoate for potentiating the "indirect" van den Bergh reaction, state that the active component is the benzoate (which can be replaced in this connexion by salicylate), while caffeine alone, and likewise caffeine-citrate, are inert. More recently Gardikas, Kench, and Wilkinson (1947) have found that, though caffeine does exert an effect, it is a relatively slow one. This last finding would appear to be consistent with the observation of Gray and Whidborne (1946) that the "indirect" reaction, using a modified Rappaport and Eichhorn technique, tended in some instances not to proceed to completion until between six and 24 hours had elapsed. Gray and Whidborne (1947) also noted that the urea present in the Rappaport and Eichhorn buffer had some effect in promoting the diazotization of bilirubin from cases of haemolytic jaundice. These workers' (1946) results for total bilirubin estimations, by their modified Rappaport and Eichhorn method, agreed well on the whole with parallel estimations by the Malloy and Evelyn method, but the readings on the sera of patients with haemolytic jaundice were made, in the case of the former method, six hours after the preparation of the test solution.

In the method described in the present paper the final mixture contains 68% (v/v) ethanol. From our own experiments with varying strengths of ethanol and from data given by Malloy and Evelyn (1937), this concentration of ethanol is certainly sufficient to produce the maximum colour. From their context it seems that the figures given by Malloy and Evelyn were derived from experiments using methanol, but they mention elsewhere in the same publication that this was preferred to ethanol only because it gave somewhat clearer solutions, and that the

development of colour was the same with ethyl alcohol. However this may be, we have found that when we have compared our method with that of Malloy and Evelyn, using sera from cases which gave an indirect reaction as judged by the classical van den Bergh test, the ethanol technique gave the higher results, the extinctions of the solutions being measured in both methods 30 minutes after their preparation.

The necessity for the further dilution of highly icteric sera, over and above the standard degree recommended for the method, is not peculiar to the present procedure. Malloy and Evelyn recommend diluting their final solution if above a level of about 15 mg. bilirubin/100 ml. of plasma, and Rappaport and Eichhorn also recommend further dilution of highly concentrated test solutions, without, however, specifying any particular ceiling value. Dilution in our method with an appropriate buffering fluid will establish the *p*H very near to that at which the optical density of the test solution corresponds exactly with that with which the artificial standard is calibrated, so that any error from this source, which would represent larger absolute amounts of bilirubin in the more jaundiced sera, will be minimized. Incidentally, any disturbing effect of the buffering capacity of the serum, which might result from the hypoproteinaemia which may accompany liver damage, will also be lessened.

The present method thus appears to us to possess certain definite advantages. Compared with the Malloy and Evelyn technique it entails less pipetting and also gives a stronger colour enabling more accurate estimations to be made on sera and plasma of relatively low bilirubin content. Moreover, the use of the artificial standard avoids the necessity of preparing a calibration curve with standard solutions of bilirubin, which is unavoidable with the Malloy and Evelyn method unless an Evelyn colorimeter is available. Compared with the Rappaport and Eichhorn procedure, the present method produces colours which are much nearer in quality to those obtained with pure bilirubin ; it employs reagents of simpler composition which are therefore easier to make up, and it enables readings to be taken at a much shorter interval after preparing the test solutions than must be allowed with the Rappaport and Eichhorn method in view of the findings of Gray and Whidborne.

The discrepancies shown in Table VI for the values yielded by the present method and that of Malloy and Evelyn are the very reverse of what might be anticipated. We had thought that " indirect bilirubin " might tend to be lost on the protein precipitate, and that methods which do not precipitate the proteins might be counted on to yield stronger colours. The fact that, on the contrary, they gave weaker colours seems to indicate either that not all the " indirect bilirubin " reacts in methanol, or that it reacts more slowly than it does in ethanol. Certainly the " indirect bilirubin " of jaundiced infants reacted very much more slowly in the glycerol mixture. The chemical and physical nature of the bilirubin, or bilirubins, of the plasma obviously needs further investigation ; but it may well be that these differences are entirely a matter of the degree of solubility of the bilirubin in different physical states. In this connexion the work of Malloy and Lowenstein (1940) is very suggestive.

The somewhat lower values given by our method for " direct bilirubin " in the obstructive jaundice cases are perhaps even more interesting (Table IV). Here it might appear that the " direct " pigment does not react as completely in ethanol as in methanol. But we find it very difficult to believe that the coupling with diazotized

sulphanilic acid does not go to completion. In our own experience, and in that of others, pure bilirubin and bile react promptly and completely in ethanol, and the colour production is complete in a few minutes. Likewise the bilirubin of sera from obstructive jaundice reacts promptly and no extra colour is obtained by allowing the reacting mixture to stand for a long time before filtering off the precipitated proteins: no more azo bilirubin can be extracted from the reacting mixture after several hours than after 30 minutes. However, the colour may be different, and greater, when the reaction is carried out in methanol in the Malloy and Evelyn method, than it is in ethanol, as is seen in Table V. But there is no greater discrepancy between the colour in methanol and ethanol with the bile diluted with serum, when there is a precipitate of proteins with the ethanol method than with the bile diluted with buffer when there is no precipitate; and therefore the lower result with ethanol cannot be due to loss of the coloured substance on the precipitate of serum proteins. We therefore think that the colour obtained in the reaction conducted in ethanol represents all the bilirubin present. The small amount of extra colour which seems to be obtained in methanol may be due to the methanol itself, as Moreland, O'Donnell, and Gast (1950) found that diazotized bilirubin developed more colour in methanol than in ethanol, or to the presence of a substance other than bilirubin (e.g., bile salts; compare Thannhauser *et al.*, 1921), which may increase the colour given by bilirubin, or may modify its quality as, indeed, often appears to be the case with the Malloy and Evelyn method where red colours with a slight bluish tint are not infrequently obtained. We hope to look for evidence on these matters by careful spectrophotometric measurements of the yellow sera from the several types of jaundice in which we have found these differences, and of the red colours they yield with the diazo reagents.

The artificial standard solution of methyl red in acetate buffer has seemed in our hands to be both the most convenient and the most reliable standard for bilirubin determinations that has been proposed. Methyl red can be obtained from almost any manufacturer of fine chemicals in a high state of purity, and it is not necessary to re-establish the relation of its colour to that of a standard prepared from pure bilirubin. All the methyl reds we have tested have yielded the same colour and have not differed from our best bilirubin standard prepared from a specimen given us by the late Professor Hans Fischer. On the other hand, the samples of so-called "pure" bilirubin which we have purchased from commercial sources have all appeared impure by reference to the Fischer bilirubin and to the methyl red standard. The bilirubins commonly used for calibrating photoelectric instruments have in our hands all appeared to be 4 to 7% impure, and results for serum bilirubin obtained by reference to such calibrations cannot be as accurate as those from comparison with the methyl red standard. For those who prefer to use a calibrated instrument with a standard graph or chart the methyl red standard furnishes an easy means for periodic checking of the standardization.

Summary

The Haslewood and King bilirubin method has been modified to yield results which are believed to represent the true bilirubin content of blood sera with a reasonable degree of accuracy.

Diazotization, coupling of the bilirubin, and precipitation of the proteins with ammonium sulphate and ethanol are performed as in the previous method ; but the reaction is carried out at a higher dilution (1 in 10 instead of 1 in 4) and the azobilirubin contained in the precipitate of proteins is included in the calculation. High bilirubin sera are diluted with buffer before diazotization in order that the final colour produced in the method may be nearly equal to that of the standard.

The coloured test solutions obtained appear to be identical with those produced from pure bilirubin. The " impure " colours frequently obtained in non-precipitation procedures are not encountered with this method.

Bilirubin added to blood sera was recovered in the method with the usual error of about 5% common to most colorimetric analyses.

Results with " indirect " sera were, on the average, somewhat higher than those obtained in parallel estimations by the Malloy and Evelyn method.

With " direct " sera from cases of obstructive jaundice somewhat lower results were obtained.

With miscellaneous sera from cases of a wide variety of clinical conditions the overall agreement was good.

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References

Gardikas, C., Kench, J. E., and Wilkinson, J. F. (1947). *Nature, Lond.*, **159**, 842.
Gray, C. H., and Whidborne, J. (1946). *Biochem. J.*, **40**, 81.
—— —— (1947). Ibid., **41**, 155.
Haslewood, G. A. D., and King, E. J. (1937). Ibid., **31**, 920.
Jendrassik, L., and Czike, A. (1928). *Z. ges. exp. Med.*, **60**, 554.
King, E. J., and Coxon, R. V. (1949). *Biochem. J.*, **44**, viii.
Malloy, H. T., and Evelyn, K. A. (1937). *J. biol. Chem.*, **119**, 481.
—— and Lowenstein, L. (1940). *Canad. med. Ass. J.*, **42**, 122.
Moreland, F. B., O'Donnell, W. W., and Gast, J. H. (1950). *Fed. Proc.*, **9**, pt. 1, 207.
Rappaport, F., and Eichhorn, F. (1943). *Lancet*, **1**, 62.
Thannhauser, J. S., and Andersen, E. (1921). *Dtsch. Arch. klin. Med.*, **137**, 179.
van den Bergh, A. A. H. (1918). *Der Gallenfarbstoff im Blute*. Leipzig. Quoted by Thannhauser and Andersen (1921).
Wootton, I. D. P. (1946). Unpublished.

J. clin. Path. (1950), 3, 260.

SERUM PROTEIN FRACTIONS: A COMPARISON OF PRECIPITATION METHODS WITH ELECTROPHORESIS

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The proteins of human serum and their changes in disease have been extensively studied by salt fractionation methods, and more recently by electrophoresis. Although there are limitations to electrophoresis (Petermann, Young, and Hogness, 1947), it has the great advantage that the albumin and globulin fractions obtained are homogeneous, although this is based upon the single property of mobility in an electric field. Unfortunately the method is not practicable for routine serum protein analysis, and a simple procedure for the chemical determination of albumin and α-, β-, and γ-globulins in serum proteins, giving results reasonably consistent with those obtained by electrophoretic analysis, would facilitate study of the alterations which occur in disease.

The so-called albumin fraction obtained after precipitation of globulin with 22.5% sodium sulphate is almost always larger than the albumin as determined by electrophoresis (Taylor and Keys, 1943 ; Dole and Braun, 1944). Petermann *et al.* (1947), reviewing the results reported in the literature obtained by both methods and also their own results, concluded that the Howe albumin fraction included α-globulin as well as albumin. This was more valid for sera from normal persons. Cohn and Wolfson (1947) arrived at the same conclusion.

Pillemer and Hutchinson (1945) reported that values for albumin in close accord with those found by electrophoresis were obtained by precipitating the globulin with aqueous methyl alcohol at *p*H 6.7–6.9, and ionic strength 0.03, providing the temperature was kept at 0° C. These results were confirmed by Martin and Morris (1949), who showed that the two methods gave values within 5% of each other. Majoor (1947), on the basis of solubility curves, found that the filtrate after precipitation with 26.8% sodium sulphate also gave values for albumin in close agreement with electrophoresis, and this was supported by the work of Milne (1947) using the same concentration of sodium sulphate. Wolfson, Cohn, Calvary, and Ichiba (1948) used 28.0% sodium sulphite for the estimation of albumin and reported good agreement.

Several precipitation methods have been advocated recently for the estimation of γ-globulin. Kibrick and Blonstein (1948) used 15.75% sodium sulphate and found agreement with electrophoresis mostly within 0.1 g., but an occasional result showed a greater difference. Jager and Nickerson (1948) precipitated the globulin

by 33% saturation with ammonium sulphate using a saturated solution brought to *p*H 7 by dilute ammonium hydroxide, obtaining good agreement with electrophoresis. Similar results were also obtained by Wolfson *et al.* (1948) using 1.89M ammonium sulphate in 4% sodium chloride solution for precipitation of γ-globulin.

The present investigation was initiated to find a reasonably simple micro-analysis of serum protein fractions by precipitation methods, giving results which would be consistent with electrophoretic analysis.

Experimental

Material.—An adult pooled human serum was obtained by mixing 1-ml. amounts of serum from 72 donors of whom 38 were Group O, 27 were Group A, six were Group B, and one was group AB. Three of the other sera were pooled infants' sera, and the remainder were from patients with a variety of conditions.

Methods.—For determining the total nitrogen in 0.1 ml. serum the micro-Kjeldahl method was used. Non-protein nitrogen was determined after precipitation of protein with 10% trichloracetic acid. In all fractions nitrogen figures were converted to protein by the factor 6.25. (All concentrations of precipitating reagents are given as initial concentrations except where otherwise stated.) The 22.5% sodium sulphate, 27.2% sodium sulphate, and 28.0% sodium sulphite solutions are made up at 37° C. and kept at this temperature until required. All precipitations and other manipulations with these reagents until the removal of the aliquot for analysis, are carried out at a temperature close to 37° C., using a warm water-bath if necessary. By this means, we have found no difficulty in using such high concentrations or reagents.

The use of ether instead of filtration, as in Kingsley's modification (1940) of the Howe method, in precipitations using sodium sulphate or sodium sulphite solutions was found to be very convenient. It is necessary to avoid vigorous shaking in the presence of ether, especially when using 27.2% sodium sulphate and 28.0% sodium sulphite, as these precipitations were found to be very sensitive in this respect. Thus we have found that if shaking is merely prolonged for 30 seconds, the precipitate of protein is increased by approximately 15%. Slight variations in other factors, e.g., temperature, grades of ether, time of centrifuging, were shown to have no effect on the precipitation.

Estimation of "Albumin plus α-Globulin" with 22.5% Sodium Sulphate.—Kingsley's modification (1940) of the Howe method was used. To 2.9 ml. of 22.5% sodium sulphate is added 0.1 ml. serum, a 2 ml. aliquot being removed for estimation by the micro-Kjeldahl method.

Estimation of Albumin with 27.2% Sodium Sulphate (Kibrick and Blonstein, 1948).—Serum, 0.1 ml., is added to 2.0 ml. of 27.2% sodium sulphate. The tube is stoppered and inverted slowly several times and then allowed to stand for one hour at 37° C. About 1 ml. ether is added, the mixture shaken gently for about 10 seconds, and then centrifuged for about three to four minutes using centrifuge cups containing warm water to avoid crystallization. Then 1.5 ml. of the lower clear aqueous layer is taken for micro-Kjeldahl determination.

Estimation of Albumin with 28.0% Sodium Sulphite (Wolfson et al., 1948).—Serum, 0.1 ml., is added to 2.4 ml. 28.0% sodium sulphite and the two mixed by inverting slowly several times. Ether, 1 ml., is added, and keeping the tube horizontal it is shaken very gently about six times and then centrifuged for three to four minutes at 3,000 r.p.m. Then 1.5 ml. of the clear lower layer is transferred for micro-Kjeldahl estimation.

No.	Subjects	Total Protein	Albumin				Albumin Plus α-Globulin		
			Electrophoresis	26%‡ Sodium Sulphate	27% Sodium Sulphite	Methyl Alcohol	Electrophoresis	21.8% Sodium Sulphate	
1	Pooled normal adult serum	7.29	3.77	3.82	3.66	3.86	5.16	4.76	
2	Beryllium poisoning ..	8.54	3.78	3.55	3.09	—	4.95	4.84	
3	Pooled infants' serum ..	6.52	3.71	3.68	3.35	3.28	4.47	4.33	
4	Cirrhosis of liver	11.47	2.16	1.50	1.83	0.92	3.23	2.68	
5	Pooled infants' serum ..	6.11	3.25	3.42	3.47	3.29	4.09	4.07	
6	Nephritis	7.54	3.23	3.24	2.81	2.57	4.24	4.55	
7	Multiple myeloma ..	11.13	2.80	2.29	1.70	0.95	4.06	3.67	
8	Normal adult serum ..	7.02	3.18	3.38	2.55	3.38	4.83	4.33	
9	Reticulosis	5.99	2.43	2.80	—	—	3.80	3.56	
10	Pooled infants' serum ..	6.01	3.86	3.57	3.67	4.27	4.80	4.17	

* Albumin as determined by methyl alcohol was actually slightly higher than the albumin and α-globulin determined by precipitati
‡ All concentrations of reagents are th

Estimation of Albumin using Methyl Alcohol (Pillemer and Hutchinson, 1945).—The method of Pillemer and Hutchinson was modified to avoid the use of a refrigerated centrifuge. All reagents and pipettes are chilled to 0° C. before use. Serum, 0.2 ml., is placed in a 10 ml. centrifuge tube which is then kept in melting ice for 15 minutes; 0.1 ml. acetic acid-sodium acetate buffer solution, followed by 0.7 ml. of the aqueous methyl alcohol, both at 0° C., are added, the tube being removed after each addition in order to mix the contents. The whole is then left in ice water for a further 30 minutes. The mixture is centrifuged until the supernatant is clear, the tubes being placed in 50-ml. centrifuge cups containing crushed ice. It was also found necessary to transfer the centrifuge tubes at the end of four minutes to fresh cups packed with crushed ice to ensure a constant temperature of 0° C. Then 0.5 ml. of the clear supernatant is removed for micro-Kjeldahl estimation.

Estimation of γ-Globulin with 15.75% Sodium Sulphate (Kibrick and Blonstein, 1948).—Serum, 0.1 ml., is added to 2.0 ml. of 15.75% sodium sulphate, mixed, and allowed to stand at 37° C. for one hour. Then 1 ml. ether is added, and the whole shaken gently. After centrifuging for three to four minutes, 1.5 ml. of the clear supernatant is transferred for nitrogen estimation by the micro-Kjeldahl method. The difference between this and the total nitrogen represents γ-globulin.

Estimation of γ-Globulin with Saturated Ammonium Sulphate Solution at pH 7.0 (Jager and Nickerson, 1948).—The method of precipitation given by these authors was followed, but the globulin was finally determined by the micro-Kjeldahl method after removal of ammonia by aeration. Precipitation is effected in a 30-ml. conical centrifuge tube drawn from glass tubing of 20 mm. bore. Serum, 0.2 ml., is used to which 0.1 ml. saturated ammonium sulphate solution is added drop by drop. A horizontal burette made from capillary tubing drawn out to a very fine jet was used to deliver the ammonium sulphate solution in at least 10 drops with mixing after each drop. This was facilitated by a thin film of vaseline applied to the outer surface of the jet. After standing overnight at 4° C., the tube is centrifuged for 30 minutes at 1,500–2,000 r.p.m. The supernatant is removed as completely as possible with a

			β-Globulin (By Difference)				γ-Globulin			
	Methyl Alcohol	Electrophoresis	15% Sodium Sulphate	33% Saturated Ammonium Sulphate	Saline Ammonium Sulphate	Electrophoresis	15% Sodium Sulphate	33% Saturated Ammonium Sulphate	Saline Ammonium Sulphate
	0.90	1.04	1.47	1.39	1.32	1.09	1.06	1.14	1.21
	—	1.66	2.00	2.07	1.72	1.93	1.70	1.63	1.98
	1.05	0.91	1.39	1.21	1.26	1.14	0.80	0.98	0.93
	1.76	†1.20	3.41	2.75	2.59	†7.04	5.38	6.04	6.20
	0.78	1.07	1.19	1.10	1.17	0.95	0.85	0.94	0.87
	1.98	0.82	0 74	0.52	Nil	2.48	2.25	2.47	2.99
	2.72	0.75	1.86	1.12	0.74	6.32	5.60	6.34	6.72
	0.95	1.17	1.68	1.47	1.25	1.02	1.01	1.22	1.44
	—	1.43	—	1.45	1.66	0.76	—	0.98	0.77
	*Nil	0.73	1.14	1.23	1.32	0.48	0.70	0.61	0.52

phate. † These results are approximations as β-globulin could not be accurately separated from γ-globulin by electrophoresis.
§ All calculations given in g./100 ml.

capillary pipette without disturbing the precipitate, and the latter washed by suspension with 0.6 ml. 33% saturated ammonium sulphate solution using a fine glass rod to effect mixing. After centrifuging for 30 minutes the supernatant is removed as before.

For the removal of ammonia, 2 ml. 2N sodium hydroxide solution is added to the precipitate and five or six drops of octyl alcohol to prevent frothing. Air freed from ammonia is now drawn through the solution in the centrifuge tube into another tube containing 1% boric acid solution and a few drops of Tashiro's indicator. A moderate current of air removes the ammonia completely in approximately 30 minutes, this being shown by the absence of colour change over a period of 10 minutes after renewal of the boric acid solution. The final solution is then transferred to a 50-ml. Kjeldahl flask for nitrogen estimation in the usual way. That there is no loss of nitrogen from the protein in this method has been shown by the analysis of known amounts of protein after the ammonia of added ammonium sulphate solution has been removed by aeration.

Estimation of γ-Globulin using Saline Ammonium Sulphate Solution (Wolfson et al., 1948).—The method of precipitation given was essentially followed, but here again the globulin was determined by micro-Kjeldahl analysis after removal of ammonia. In order to avoid transfer, the globulin is precipitated in a 30-ml. centrifuge tube by the addition of 0.2 ml. serum to 4.8 ml. of the saline ammonium sulphate solution and mixed by slow inversion for one to two minutes. After centrifuging for 30 minutes at 3,000 r.p.m. the clear supernatant is removed by decantation from the closely packed precipitate, the tube inverted and allowed to drain for 10 minutes. The precipitate is dissolved in 2 ml. 2N sodium hydroxide solution, care being taken to wash the sides of the tube, and then five to six drops of octyl alcohol are introduced. Ammonia is removed by aeration and the nitrogen estimated by micro-Kjeldahl analysis as before.

Results

The results of the analysis of 10 different sera are shown in the table, in which concentrations stated are the final, and not the initial, concentrations of reagents.

Duplicate analyses were always performed and the means taken ; in all methods there was no difficulty in getting good duplicate results. As indicated below, α-globulin was calculated by the difference between the fraction obtained by precipitation with 22.5% sodium sulphate and the albumin as determined by 27.2% sodium sulphate, 28.0% sodium sulphite, and methyl alcohol precipitation respectively. β- and γ-Globulin together was calculated by the difference between the total protein and the fraction obtained by 22.5% sodium sulphate. The difference between this and the experimentally determined γ-globulin gave β-globulin. Electrophoresis (by Dr. H. Hoch) was carried out using a veronal buffer at *p*H 8.6 and ionic strength 0.15, except for serum 4 when phosphate buffer *p*H 7.4 and ionic strength 0.1 was used.

Discussion

In general it may be stated that the albumin and globulin fractions obtained by precipitation methods and by electrophoresis could not be expected to give identical results ; if these were obtained, it could only be fortuitous, due to the inclusion of a certain amount of another fraction being estimated.

Albumin and α-Globulin.—The results using 22.5% sodium sulphate support the conclusion of Petermann *et al.* (1947) that this albumin fraction includes α-globulin as well as albumin. The agreement between this fraction and the albumin and α-globulin found by electrophoresis is reasonably good, the difference in most cases being not more than 0.4 g. %.

Albumin.—Of the three methods, 27.2% sodium sulphate gave the most satisfactory results for all sera, the mean deviation from the electrophoresis results being 9% compared with 15% and 22% for 28.0% sodium sulphite and methyl alcohol respectively. For normal sera agreement between precipitation methods and electrophoresis is much closer, the mean deviation being 6%, 9%, and 6% for 27.2% sodium sulphate, 28.0% sodium sulphite, and methyl alcohol respectively. The results we have obtained with pathological sera using methyl alcohol have not been so good as those reported by other investigators, in every case very low values being obtained. It was found in the latter part of the investigation that the sodium sulphite solution deteriorates, and should be made up freshly each week. Some of the low results shown in the table may possibly be due to this, since old solutions were found to give low results.

γ-Globulin.—A comparison of the three methods used with electrophoresis shows that all give reasonably good agreement for both normal and pathological sera, the mean deviation being 16% for 15.75% sodium sulphate and 12% for both the ammonium sulphate methods. As might be expected, the sodium sulphate method agrees less well with electrophoresis than the ammonium sulphate methods, since in the former the γ-globulin is estimated by difference. Thus, a small experimental error in the determination of nitrogen in the supernatant after precipitation of the globulin will be magnified into much greater errors in the γ-globulin. Even though there is little to choose between the other two methods, 33% saturation with ammonium sulphate gives a slightly better spread of deviation from the electrophoresis results. However, this method is technically more difficult and requires overnight precipitation. The difference between the two methods is not sufficiently significant to make a choice.

The experimental results indicate that for the determination of true albumin the best method is 27.2% sodium sulphate, and for γ-globulin either saline or saturated ammonium sulphate. If, as our results and those of previous investigators show, the albumin fraction determined by 22.5% sodium sulphate may be taken as albumin plus α-globulin, the value of α- and β-globulin may be determined by difference. This investigation shows that it is quite feasible to estimate all the fractions by precipitation methods in 0.5 ml. serum with results which agree sufficiently closely with electrophoresis to enable chemical methods to be used for the study of alterations in the pattern of serum proteins occurring in disease, or to follow quantitatively the changes which take place during progress or treatment.

Summary

Several precipitation methods for the estimation of true albumin and of γ-globulin have been modified for small amounts of serum and the final analysis made by the micro-Kjeldahl method, and the results obtained compared with electrophoresis. The selected methods for a number of normal and pathological sera gave results in good agreement with those obtained by electrophoresis. It is thus possible to carry out an analysis of serum proteins for albumin and α-, β-, and γ-globulins by chemical precipitation methods using only 0.5 ml. serum.

We wish to thank Dr. H. Hoch for carrying out the electrophoretic analyses on the sera. The above forms part of a larger investigation on serum protein levels of infants undertaken by B. Levin, Helen M. Mackay (member of the scientific staff of the Medical Research Council), and Catherine A. Neill, with the assistance of V. G. Oberholzer and T. P. Whitehead, who gratefully acknowledge grants from the Medical Research Council.

References

Cohn, C., and Wolfson, W. Q. (1947). *J. Lab. clin. Med.*, **32**, 1203.
Dole, V. P., and Braun, E. (1944). *J. clin. Invest.*, **23**, 708.
Jager, B. V., and Nickerson, M. (1948). *J. biol. Chem.*, **173**, 683.
Kibrick, A. C., and Blonstein, M. (1948). Ibid., **176**, 983.
Kingsley, G. R. (1940). Ibid., **133**, 731.
Majoor, C. L. H. (1947). Ibid., **169**, 583.
Martin, N. H., and Morris, R. (1949). *J. clin. Path.*, **2**, 64.
Milne, J. (1947). *J. biol. Chem.*, **169**, 595.
Petermann, M. L., Young, N. F., and Hogness, K. R. (1947). Ibid., **169**, 379.
Pillemer, L., and Hutchinson, M. C. (1945). Ibid., **158**, 299.
Taylor, H. L., and Keys, A. (1943). Ibid., **148**, 379.
Wolfson, W. Q., Cohn, C., Calvary, E., and Ichiba, F. (1948). *Amer. J. clin. Path.*, **18**, 723.

J. clin. Path. (1950), 3, 266.

THE FRACTIONATION OF SERUM PROTEINS USING SODIUM SULPHATE

BY

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Before the development of the moving boundary technique of electrophoresis it had been the custom to classify the circulating serum proteins into three broad groups, albumin, pseudoglobulin, and euglobulins, based on their solubilities in salt solutions of varying concentrations. Howe (1921), using sodium sulphate in varying concentrations, defined three zones in which there was little or no alteration in the amount of protein precipitated. These lay between 13.5 and 14.5%, 16.4 and 17.4%, and between 21 and 22%. The technical developments in electrophoretic analysis associated with the name of Tiselius (1939) have popularized the classification of the serum proteins on the basis of their mobilities in an electrical field, the broad groups being designated as albumin and α, β, and γ globulin.

Majoor (1947) carried out a careful study of the solubility of serum proteins over a wide range of concentrations of sodium sulphate and compared these with electrophoretic analysis. Four sera were examined: one normal, one from a patient suffering from chronic hepatitis, one from a patient suffering from myeloma, and one from a patient suffering from chronic nephritis. A study of his curves demonstrates zones of precipitation occurring between concentrations of sodium sulphate of 14 to 18 g. per 100 ml. of water, 18 and 26 g. per 100 ml., and between 26 and 34 g. per 100 ml. As a result of his investigations Majoor has suggested that concentrations of 18.5 g. sodium sulphate per 100 ml. water and 26.8 g. sodium sulphate per 100 ml. water would give a convenient and sufficiently characteristic division of the serum proteins for routine clinical use. Such a procedure gives three fractions, the most soluble being almost pure albumin, the next most soluble largely α and β globulins, and the least soluble principally γ globulin. Majoor admits that there is an overlap of the three fractions and that no absolute separation is obtained by such a simple procedure. Milne in 1947 examined 11 sera from different diseases using 26.8 g. sodium sulphate to 100 ml. water and 19.6 g. sodium sulphate to 100 ml. water. These concentrations, he claims, give separation into three fractions corresponding to albumin, the α globulins, and the combined β and γ globulins. His analyses show remarkably good agreement, but it is noteworthy that no sera are included from patients suffering from nephritis, in which increases in the α and β globulins are frequently observed. Kibrick and Blonstein (1948) have used 15.75 g. sodium sulphate per 100 ml. water to precipitate γ globulin but noticed occasional gross deviations from the electrophoretic analysis. Over the past three years studies have been continuing in this laboratory on convenient means of protein fractionation and their application to the study of disease. The data analysed here were collected

in part as an ancillary to a study reported by Martin and Morris (1949) on the precipitation of total globulin. Two objectives were borne in mind: first, could a reasonable correlation be obtained between analysis by electrophoresis and analysis by simple salt fractionation over a diverse range of clinical conditions, and, second, could a simple fractionation yield material suitable for further detailed study ?

Material and Methods

Venous blood was drawn, under paraffin, from fasting patients suffering from a variety of clinical conditions, into clean centrifuge tubes without any anticoagulant. It was allowed to clot and then spun at 2,500 revolutions, the clear serum being withdrawn for analysis. In each instance the electrophoretic analysis and salt fractionations were all carried out on one serum sample.

The electrophoretic analyses were carried out in the Tiselius apparatus under the same conditions as detailed in an earlier paper (Martin and Morris, 1949). Precipitation of the globulins was carried out with precautions outlined in the same paper. The concentrations of sodium sulphate used are listed in Table I.

TABLE I

CONCENTRATIONS OF SODIUM SULPHATE

	Concentration (g./litre)	Approximate Final Molar Concentration before Precipitation	Temperature	Range of Protein Concentrations (g. per 100 ml.)	Precipitation Time (Hours)
Sodium sulphate:					
Na_2SO_4*	130	0.9	37° C.	0.14–0.29	24
Na_2SO_4	150	1.06	37° C.	0.14–0.29	24
Na_2SO_4	190	1.34	37° C.	0.14–0.29	24
Na_2SO_4	260	1.77	37° C.	0.14–0.29	24

* In each instance the proportion of serum to sodium sulphate solution was 1 to 29.

Results

The results of 10 analyses are shown in Table II. In the table the electrophoretic analyses have been summed so that direct comparison may be made with salt fractionation. There is reasonable agreement between the residual soluble protein after precipitation with 26% sodium sulphate and albumin determined electrophoretically. Analysis of the supernatant after precipitation with 19% sodium sulphate gives a reasonable approximation to the electrophoretic values for the sum of albumin and α globulin in seven out of the ten sera ; in the other three, all from patients suffering from nephritis, there is a gross discrepancy. Analyses of the supernatant after precipitation with 15% sodium sulphate agree well with electrophoretic values for the sum of albumin and α and β globulins in sera 3 and 6, while analysis after precipitation with 13% sodium sulphate agree well for sera 1, 4, and 7. In sera 8, 9, and 10, all from cases of nephritis, there is little agreement, salt fractionations usually giving higher figures for γ globulin than those arrived at by electrophoresis. In serum 2, from a case of hepatitis, the mean value of the two salt fractionations gives a good approximation to the γ globulin content estimated by electrophoresis. In serum 5 both salt fractions give low values for γ globulin compared with electrophoresis.

TABLE II

COMPARISON OF ELECTROPHORETIC AND SODIUM SULPHATE ANALYSIS

No.	Disease	Serum	Analysis by Electrophoresis			Soluble Protein after Precipitation with Sodium Sulphate (g./100 ml.)				γ Globulin by Electrophoresis	γ Globulin by Difference (15% Sod. Sulphate Precipitation)	γ Globulin by Difference (13% Sod. Sulphate Precipitation)
		Total Protein (g./100 ml.)	Albumin	Albumin + α Globulin	Albumin + α and β Globulin	26%	19%	15%	13%			
1	Hepatitis	7.8	2.85	5.45	6.8	2.8	5.4	6.6	6.8	1.05	1.2	1.05
2	Hepatitis	7.2	2.0	5.0	6.0	2.1	4.8	5.7	6.2	1.2	1.5	1.0
3	Cirrhosis	6.4	1.47	2.09	3.10	1.45	2.05	3.0	3.2	3.3	3.4	3.2
4	Cirrhosis	7.6	2.8	3.35	4.85	2.7	3.4	4.5	4.7	2.75	3.1	2.9
5	Cirrhosis	8.2	2.0	2.6	3.4	1.9	2.55	3.85	5.28	4.8	4.35	2.92
6	Myeloma diffuse	8.5	1.06	1.08	1.95	1.15	1.2	2.0	2.5	6.55	6.5	6.0
7	Myeloma solitary	5.1	2.3	3.22	4.00	2.1	3.3	3.8	4.0	1.1	1.3	1.1
8	Nephritis	4.4	0.98	3.25	3.77	0.96	1.55	2.9	3.4	0.63	1.5	1.0
9	Nephritis	4.6	0.4	1.6	2.8	0.35	1.9	2.1	3.35	1.8	2.5	1.25
10	Nephritis	6.23	2.1	4.8	5.2	2.39	4.35	4.5	5.4	1.03	1.73	0.85

Discussion

Majoor (1947) and Milne (1947) have both studied the problem in normal and a limited number of abnormal sera. The present series deals entirely with abnormal sera. It demonstrates that while reasonable correlation exists between albumin determined electrophoretically and determined by salt precipitation, the correlation of the globulin fractions is a more difficult matter. Milne showed good agreement between analysis obtained by precipitation with 19.6% sodium sulphate and the electrophoretic analysis of albumin and α globulin combined. Our figures using 19.0% sodium sulphate also show reasonable agreement with the exception of the sera from patients with nephritis, where there are gross discrepancies. Milne's series includes no such cases. It is precisely these cases, in which there is a marked increase in the α and β globulins, that form a critical test of the procedure. The deviation is not unexpected when it is recalled that electrophoretic analyses by the Tiselius technique are dependent on the refractive increment, whereas the analyses of salt fractionation are based on nitrogen determinations. Armstrong, Budka, and Morrison (1947), in an exhaustive study of material isolated by the more elaborate techniques of cold alcohol fractionation (Cohn, Strong, Hughes, Mulford, Ashworth, Melin, and Taylor, 1946), have shown the extent to which commonly accepted nitrogen factors must be corrected when dealing with concentrations of individual serum protein fractions, especially when, as in the case of α and β globulins, they contain a relatively high percentage of lipid.

Estimations of the γ globulins are not complicated by this factor to the same extent. The γ globulins, however, form a large and complex family. Electrophoretic analysis of sera from patients suffering from advanced, long-standing liver damage repeatedly shows the "γ globulin" component as a peak with a wide base suggesting a group of closely associated proteins rather than an excess of an individual protein (Thorn, Armstrong, and Davenport, 1946; Martin, 1949; Sterling and Ricketts, 1949). This is in contrast to the typical analysis of diffuse myelomata in which the excess of γ globulin in a given serum occurs as a well-defined peak of uniform mobility. As might be anticipated analysis of such a serum (No. 6) using 26% sodium sulphate fractionation and 15% sodium sulphate fractionation gives a working approximation of the chief features of the distribution of the serum proteins compared with electrophoresis. The precision is not mirrored in the analysis of the sera from cirrhotics, though a good working comparison may be obtained by using sulphate concentrations of 15 to 13%.

It is evident from our results, as from those of Majoor and Milne, that simple fractionation procedures can be evolved which give an approximation to electrophoretic analysis sufficient for many routine purposes, but that they must always be used with caution. Their correlation will tend to break down in any case where there is gross excess of α or β globulins or of atypical lipoproteins. Good correlation is not to be expected therefore in sera from patients with conditions such as subacute nephritis or leptospirosis. Provided these limitations are borne in mind and provided always that the method is treated as an approximation there can be no objection to its use.

In this paper we deliberately chose sodium sulphate concentrations differing from those used by Majoor, by Milne, and by Kibrick and Blonstein, so that workers

interested might have observations over a range of concentrations. Milne has used 19.6% sodium sulphate and Majoor 18.5% sodium sulphate. Majoor maintains that at this concentration albumin and α and β globulin remain in solution. We think that for many abnormal sera 18.5% sodium sulphate is too high a concentration to give the optimum point of separation of α and β globulins. We are equally certain that with 13% sodium sulphate a significant amount of γ globulin remains in solution with the β globulin in many abnormal sera. We have favoured 15% sodium sulphate as striking a balance between the two levels, but it is clear that even at this level one may get marked discrepancies with some sera. We have not satisfied ourselves that the method of sodium sulphate fractionation is a convenient one for preparing material for further detailed study of either albumin or globulin fractions. We have obtained globulin by precipitation with 13% sodium sulphate which could be brought smoothly into solution, but this does not happen in every instance. The α and β globulins have proved even more difficult to handle by sodium sulphate precipitation. The preparation of albumins for detailed study by sodium sulphate fractionation we found objectionable for two reasons: the necessary 30 times dilution of the serum and the introduction of a salt solution more than 10 times the molarity of physiological saline. This entails prolonged dialysis and subsequent concentration before the albumin is in convenient concentration for further detailed study. The dilution is not objectionable if chromatographic studies of amino-acids are contemplated, though dialysis to reduce the high salt concentration would still be necessary even for these studies.

Simple fractionation of serum proteins using 26% and 15% sodium sulphate when used with discretion does offer an approximation to the broad electrophoretic distribution of serum proteins in a number of conditions. It breaks down absolutely in the study of sera from patients suffering from subacute nephritis and may be expected to be of little value where more precise differentiation of the α and β globulins is required.

References

Armstrong, S. H., jun., Budka, M. J. E., and Morrison, K. C. (1947). *J. Amer. chem. Soc.*, **69**, 416.
Cohn, E. J., Strong, L. E., Hughes, W. L., jun., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L. (1946). Ibid., **68**, 459.
Howe, P. E. (1921). *J. biol. Chem.*, **49**, 115.
—— (1923). Ibid., **57**, 235, 241.
Kibrick, A. C., and Blonstein, M. (1948). Ibid., **176**, 983.
Majoor, C. L. H. (1947). Ibid., **169**, 583.
Martin, N. H. (1949). *Brit. J. exp. Path.*, **30**, 231.
—— and Morris, R. (1949). *J. clin. Path.*, **2**, 64.
Milne, J. (1947). *J. biol. Chem.*, **169**, 595.
Sterling, K., and Ricketts, W. E. (1949). *J. clin. Invest.*, **27**, 1469.
Thorn, G. W., Armstrong, S. H., jun., and Davenport, V. D. (1946). Ibid., **25**, 304.
Tiselius, A. (1939). *Harvey Lect.*, **35**, 37.

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THE RENAL THRESHOLD FOR BILIRUBIN

BY

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There appears to be scanty evidence as to the exact renal threshold for bilirubin. Van den Bergh (1928) states that it is 1/50,000 (2 mg./100 ml.) with remarkably few exceptions, but does not say how exact this estimate is, apparently basing his statement on numerous disconnected observations. This figure appears to be quoted by others.

Weiss (1935) observes: "It is stated that bile does not appear in the urine until four units (i.e., 2 mg./100 ml.) are present in the blood. In haemolytic jaundice this relation does not hold, since it is quite possible to have between five and 18 units present in the blood with no bile in the urine." Whitby and Britton (1946) quote the same figures, and Harrison (1947) quotes Weiss directly, but suggests that the use of sensitive urine tests would modify his claims for the threshold value, and that this can be 0.5–1.0 mg./100 ml. With (1943), on the other hand, states that the threshold value lies between 2 and 6 mg./100 ml., with considerable variation.

An opportunity to investigate the question occurred during an epidemic of infective hepatitis among Service patients in a military hospital. As soon as a clinical diagnosis was made twice-weekly estimations of plasma bilirubin content were made by the van den Bergh method and the Lovibond comparator (to the nearest 0.2 mg./100 ml.) on 53 patients, together with simultaneous urine tests by Fouchet's method for bile. Although this is not quite the most sensitive method, it is not considered that its use has led to any material error in the final conclusions.

Method

The plasma bilirubin level at the time when bile was last found in the urine during recovery from the condition was noted, and for convenience was called the "above threshold" value. Similarly the plasma bilirubin level when bilirubin first ceased to be found in the urine during recovery was noted and called the "below threshold" level. It was argued that the true renal threshold must lie between these two levels. The number of occasions on which the various values for these two levels were found have been plotted as superimposed frequency diagrams in Fig. 1.

Discussion

The figure shows two significant features: (1) the definite modal values, and (2) the pronounced scatter of values.

Modal Values.—There are definite modal values of 1.0 and 0.8 mg./100 ml. for the "above" (13 cases) and "below" (18 cases) threshold levels respectively,

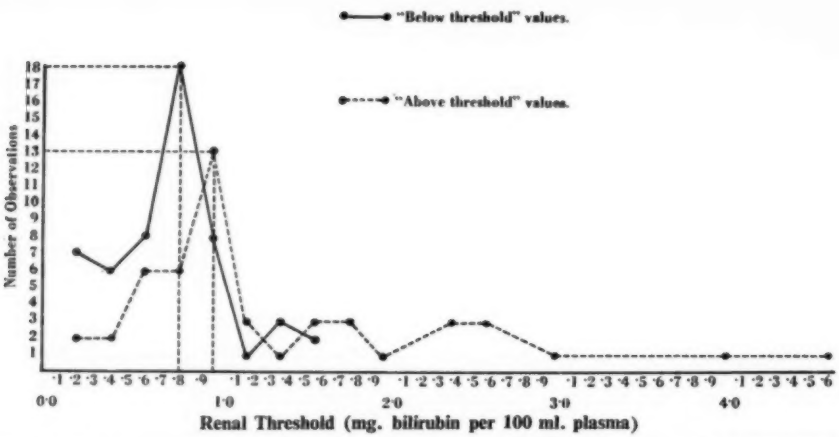

FIG. 1.—Superimposed frequency diagrams of 53 observations of bilirubin levels. "Below threshold" values show a standard deviation of 0.11, a mean of 0.71, and a variance of 15%. "Above threshold" values show a standard deviation of 0.93, a mean of 1.32, and a variance of 70%.

which strongly suggest that the renal threshold itself has a modal value between those levels. Both levels were recorded in the same patient on six occasions. That this did not happen more often is probably due to the fact that the rate of fall of plasma bilirubin was often too fast for the exact recording of the two levels in the same patient by the method adopted.

Scatter of Values.—This is marked and could have been due to the following factors.

True Variation in the Renal Threshold.—This possibility was suggested by the observation that, in certain cases, urinary excretion of bilirubin apparently ceased while the plasma bilirubin level remained constant or even rose slightly. A very similar phenomenon was noted by With (1943). This mechanism could account for the scatter immediately around the modes.

Variation in the Chemical Nature of Bilirubin.—It is of interest that Vaughan and Haslewood (1938), when attempting to estimate normal bilirubin levels, found a coefficient of variation of 45.93%, which is of the same order as those found in the present investigation (70 and 15%). It is not certain whether this was entirely due to variations between the persons investigated or partially to unrecognized difficulties in estimating bilirubin. That there may be unrecognized difficulties in estimating bilirubin was shown by Weiss (1935) when he drew attention to the inconstant relationship between serum bilirubin and the icterus index. The effect of any such factors on the present investigation need not be elaborated.

A well-recognized difference exists between the nature of "direct" and "indirect" bilirubin, and each is known to have its own renal threshold, which in the latter case is very variable. This has been mentioned indirectly in Weiss's statement which I have quoted. In fact, as With (1943) points out, it may be misleading to think of bilirubin as a true threshold substance at all. Both forms of bilirubin occur in the

plasma of infective hepatitis and it is considered that this is the main cause of the present findings. Those cases with a predominantly "direct" bilirubinaemia showed the modal threshold and neighbouring levels, whereas those cases with an "indirect" bilirubinaemia showed the variety of much higher threshold levels.

Summary

There is no one renal threshold level for bilirubin. There is a modal level, seen in a minority of cases, which lies between plasma bilirubin levels of 0.8 and 1.0 mg./100 ml. This and similar low levels occur in "direct" bilirubinaemia. A variety of much higher levels may occur in "indirect" bilirubinaemia. True variations in the threshold may occur in the same person independently of the type of bilirubin excreted.

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REFERENCES

Bergh, A. A. Hijmans van den (1928). *Der Gallenfarbstoff im Blute*, 2nd ed., p. 27. Leiden and Leipzig.
Harrison, G. A. (1947). *Chemical Methods in Clinical Medicine*, 3rd ed., p. 241. London.
Vaughan, J. M., and Haslewood, G. A. D. (1938). *Lancet*, **1**, 133.
Weiss, S. (1935). *Diseases of the Liver, Gall Bladder, Ducts, and Pancreas*, p. 166. New York.
Whitby, L. E. H., and Britton, C. J. C. (1946). *Disorders of the Blood*, 5th ed., p. 598. London.
With, T. K. (1943). *Acta med. scand.*, **114**, 426.

J. clin. Path. (1950), 3, 274.

THE NATURE OF THE TISSUE FLUID IN THE OEDEMA OF TOXAEMIA OF PREGNANCY

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Very little is known about the nature of the tissue fluid in the oedema of pregnancy toxaemia. Most investigators (Dieckmann, 1942 ; Taylor, Warner, and Welsh, 1939 ; Chesley and Chesley, 1943) believe that the abnormal water balance in this condition is caused by a combination of several factors, both mechanical and physico-chemical in nature. There is general acceptance of the fact that the oedema in toxaemias of pregnancy is brought about by a retention of sodium chloride in the tissues, and some observers (Taylor *et al.*, 1939) suggest that this is connected with an oestrogen excess which accompanies pregnancy. No attempt has, however, been made to study the properties of the oedema fluid and to determine if in pre-eclamptic oedema the normal physico-chemical equilibrium between the plasma and tissue fluid is in any way disturbed.

Materials and Method

The present investigation is based upon the chemical analysis of the plasma and tissue fluid collected from 45 cases of pre-eclampsia with varying degrees of oedema.

The tissue fluid was obtained from the subcutaneous tissue of the oedematous limb (usually the inferior extremity) with a No. 11 hypodermic needle. Before the fluid for analysis was collected in a test-tube the needle was connected to a water manometer to determine the tissue fluid pressure. Throughout the whole procedure care was taken to avoid any pressure not only on the part itself but also on the veins draining the limb, which was kept at rest at the cardiac level. Blood for analysis was obtained from the cubital vein at the same time as the tissue fluid. A portion of the tissue fluid and blood was collected under liquid paraffin for the estimation of bicarbonate. To evaluate the results obtained in toxaemias of pregnancy, values for normal pregnancy must be known. The difficulty in obtaining the subcutaneous tissue fluid in non-oedematous subjects is obvious and to overcome it samples of peritoneal fluid were obtained from six cases of normal pregnancy during caesarean section. In each case the specimen was collected from the anterior pelvic compartment immediately after laparotomy, care being taken to avoid spilling blood into the peritoneal cavity. The quantity of fluid obtained in this manner was small but was found sufficient for analytical purposes.

Tissue tension in normal pregnancy was measured by the same method as that employed for cases of toxaemia.

Standard methods (King, 1946 ; Peters and Van Slyke, 1932) for microanalysis were employed for all chemical estimations. Each estimation was done in duplicate and the results for all mineral constituents were expressed in milli-equivalents per litre.

For the purposes of this description our cases of pre-eclamptic oedema have been divided into four groups. Group 1 consists of six cases with slight oedema confined to the feet and ankles only; Group 2, of 11 cases with pitting oedema of the feet, ankles, and legs; Group 3, of 12 cases with marked oedema of the inferior extremities with or without slight puffiness of the face and hands; and Group 4 contains 16 cases with generalized oedema.

Physical Characteristics of the Tissue Fluid

Both normal and oedematous tissue fluids are crystal clear in appearance. On standing for several hours a delicate, woolly coagulum forms. Unlike a fibrinous clot it does not retract even on prolonged standing. Admixture with a small quantity of fresh plasma or serum causes a marked reduction in clotting time and the clot is heavier and more retractile. The cellular constituents are scanty in both normal and toxaemic cases. Not more than 8 lymphocytes per c.mm. were found in either.

Tissue Tension

The effect of tissue tension in oedema has been studied by several investigators. Loeb (1923) suggested that a diminution in tissue tension might be responsible for the occurrence of oedema. Dieckmann (1942), on the other hand, observed that in the oedema of pre-eclampsia the tissue tension was increased.

We measured the tissue tension in a limb at cardiac level in 25 cases of normal pregnancy and in 45 patients suffering from pre-eclampsia. The results are shown in Table I.

TABLE I

TISSUE TENSION IN NORMAL PREGNANCY AND PREGNANCY TOXAEMIA

	Normal Pregnancy	Pre-eclampsia			
		Group 1	Group 2	Group 3	Group 4
Oedema	0				
No. of cases	6	6	11	12	16
Tissue tension (mm. H_2O): *Minimum*	13.6	15.5	18.8	23.8	24.9
Maximum	21.0	26.3	30.0	34.0	34.7
Average	18.25	19.7	22.7	26.6	30.1
Standard deviation ..	1.74	4.17	3.13	2.96	2.49
Significance		—	+	+	+

It is evident that as oedema increases the tissue tension tends to rise. In generalized oedema the coefficient of variation of the values for tissue tension diminishes and is less than that in normal pregnancy. In the various degrees of oedema, however, there is considerable overlapping. From the time oedema becomes appreciable the maximum figures show little variation in the series, but the minimum values tend to rise.

This increase in tissue tension, in the absence of other factors, will tend to reduce the effective filtration pressure and thereby limit the degree of oedema. In toxaemia, however, visible oedema does not affect loose areolar tissue (e.g., eyelids) until the condition is severe and extensive, although tissue tension is less in these regions. Progressive oedema with increasing tissue tension, in the absence of an active fluid-shift, presupposes a progressively rising intracapillary pressure. It has already been shown that the level of blood pressure bears no consistent relationship with oedema (Mukherjee and Govan, 1950).

When the limb is subjected to the effects of gravity the tension within the tissues increases considerably. In normal pregnancy the tissue pressure was raised by 52.5% after 20 minutes. In patients with pre-eclamptic oedema a similar treatment caused the tension to increase by 106.2% of the basic level. This is in agreement with an observation made by Burch and Sodeman in 1938, who found that venous compression of a limb in a normal subject may raise the tissue pressure by 20 mm. The average increase in tissue tension caused by the effects of gravity for 20 minutes was 9.3 ± 2.1 mm. in normal pregnancy and 29.1 ± 3.3 in pre-eclamptic oedema. It is of interest to know that in all toxaemic patients gravity caused the tissue pressure to rise above the normal intracapillary pressure. However, no correlation was found between the tissue tension and the brachial arterial pressure in any instance.

Organic Constituents of Tissue Fluids

Protein.—Tissue fluid was consistently found to be poor in protein in all cases. In six normal cases it was found to contain only 0.2 g.% of protein with a range of values between 0.27 and 0.16 g. per 100 ml. The protein content of the fluid bore no relationship to that of the plasma.

In 26 cases of toxaemia of varying severity the average protein concentration of the tissue fluid was 0.24 g. per 100 ml. The maximum and minimum values were 0.32 and 0.17 g. per 100 ml. respectively. The difference between the averages of normal and toxaemic cases had no statistical significance.

Estimation of the individual protein fractions was undertaken in two normal and 12 toxaemic cases. The globulin content was low in all instances, although it showed a slight variation relative to the total protein content of the fluid. As a result the albumin : globulin ratio was fairly constant. The results are given in Table II.

The albumin : globulin ratio was alike in both normal and toxaemic pregnancy and considerably higher than that of the plasma. No relationship could be found between the ratio for tissue fluid and that of the corresponding plasma, nor did the variation in the ratio correspond with the degree of oedema.

Only a trace of fibrinogen could be found in the tissue fluid of both normal and toxaemic cases. Indirect estimations using thromboplastin (Russell viper venom) were made in seven patients, the difference in nitrogen content of the fluid before and after clotting being regarded as due to fibrinogen. The results varied from 1 to 3% of the total protein. Gravity was not found to alter the protein content of the tissue fluid.

The protein content of the tissue fluid in pre-eclamptic oedema has been studied by other observers (Hellmuth, 1922 ; Albano, 1927) and our findings corroborate their results.

TABLE II

PROTEINS OF PLASMA AND TISSUE FLUID IN NORMAL AND TOXAEMIC PREGNANCY

	Patient	Maturity (weeks)	Blood Pressure (mm. Hg)	Albumin (g./litre)	Oedema	Tissue Fluid		Plasma
						Protein (g. %)	A/G Ratio	A/G Ratio
Normal	1 O. ..	37	120/70	0	–	0.21	2.14	1.51
	2 S. ..	39	116/72	0	–	0.17	2.11	1.49
Toxaemic	1 McG.	36	150/100	3	++	0.19	2.06	1.10
	2 St. ..	36	170/104	8	+++	0.23	2.13	1.23
	3 C. ..	34	160/100	2	++	0.20	2.11	1.27
	4 T. ..	39	160/105	4	+++	0.34	2.12	1.05
	5 K. ..	32	180/110	10	+++	0.18	2.10	1.17
	6 N. ..	34	175/100	8	++	0.29	2.09	1.24
	7 McA.	37	165/100	3	++	0.23	2.12	1.22
	8 McN.	40	185/120	6	+++	0.19	2.14	1.06
	9 O'R.	33	165/105	5	++	0.24	2.09	1.14
	10 H. ..	36	178/120	6	+++	0.17	2.10	1.13
	11 B. ..	36	150/102	4	++	0.21	2.13	1.26
	12 G. ..	40	168/105	5	+++	0.28	2.13	1.11
					Average (toxaemic)	0.23	2.12	1.16
					Standard deviation (toxaemic)	0.07	0.02	0.085

Urea, Non-protein Nitrogen, Creatinine, Uric Acid, and Sugar.—These substances were found in identical concentration in plasma and tissue fluid. Our results obtained in normal and toxaemic pregnancy are presented in Table III.

In view of the freely diffusible nature of these substances it is reasonable to expect that they would be equally distributed between plasma and tissue fluid. Dieckmann (1942), however, has stated that the tissue fluid is probably richer in urea than blood. Findings in this laboratory do not substantiate this, but they agree with those of other authors' investigations on experimental oedema and ascites (Greene, Bollman, Keith, and Wakefield, 1931; Hastings, Salvesen, Sendroy, and Van Slyke, 1927).

TABLE III

ORGANIC CONSTITUENTS OF PLASMA AND TISSUE FLUID

	Normal Pregnancy (6 Cases)		Pre-eclampsia (26 Cases)	
	Plasma	Tissue Fluid	Plasma	Tissue Fluid
Urea	26.0±1.43*†	26.0±1.43	22.4±3.28	22.3±3.60
	20.0–29.0	20.0–29.0	15.2–36.8	16.1–37.0
N.P.N.	28.3±1.39	28.2±1.40	32.5±1.54	32.5±1.54
	23.1–33.2	22.6–33.0	22.6–40.0	23.3–39.3
Creatinine	1.24±0.094	1.25±0.087	1.25±0.094	1.26±0.087
	1.10–1.58	1.10–1.58	1.16–1.60	1.20–1.56
Uric acid	3.79±0.41	3.80±0.41	5.0±0.39	5.0±0.39
	3.0–4.45	3.0–4.43	4.38–6.10	4.40–6.0
Sugar	91.4±4.25	91.5±4.72	82.4±4.01	82.4±2.80
	86.2–98.3	86.0–97.8	78.6–94.0	79.4–94.0

* All results are expressed in mg./100 ml.

† The above figures in each case represent the average together with the probable error and the minimum and maximal values.

Cholesterol and Phospholipoids.—The concentration of the cholesterol in the plasma was higher than that of tissue fluid in both normal and toxaemic cases, and no correlation could be found between the two levels. The average plasma cholesterol level in normal pregnancy was 238.6 mg. per 100 ml. and that of the tissue fluid 66.4 mg. per 100 ml. In pre-eclampsia the plasma cholesterol level increased to 271.6 mg. per 100 ml., but the tissue fluid cholesterol level was only 65.9 mg. per 100 ml. The increase in the plasma cholesterol level thus appears to have no effect on the value for tissue fluid. The highest tissue fluid cholesterol level observed was 83.5 mg. per 100 ml., and this was in a patient who was only moderately toxic, with a plasma cholesterol level of 242 mg. per 100 ml.

The behaviour of lipoid phosphorus was in striking contrast to that of cholesterol. In normal pregnancy the lipoid phosphorus was almost equally distributed between plasma and tissue fluid, the average values being 12.7 mg. per 100 ml. and 12.6 mg. per 100 ml. respectively. In toxaemia, however, the average value for the tissue fluid lipoid phosphorus was 20.8 mg. per 100 ml. while that of the plasma was 14.5 mg. per 100 ml. The difference between these two values was statistically significant. The change was not confined to the average but was reflected also in the maximum and minimum values. It has been suggested (Boyd, 1935) that the retention of phospholipoids, because of their hydrophilic properties, might be responsible for the accumulation of oedema fluid. The results have therefore been analysed in relation to the degree of clinical oedema, and are shown in Table IV. It is obvious from our findings that although the tissue fluid lipoid phosphorus is raised in oedematous patients the value does not bear any consistent relationship with the severity of the oedema.

TABLE IV

PHOSPHOLIPOIDS OF TISSUE FLUID

	Normal Pregnancy	Pre-eclamptic Oedema		
		Group 2	Group 3	Group 4
No. of cases	6	4	11	11
Minimum	9.5*	16.6	17.0	19.7
Maximum	15.9	21.3	23.0	24.0
Average	12.7	19.6	20.1	22.0
Standard deviation ..	1.66	2.27	1.83	1.50
Significance		+	−	+

* All results expressed as mg. lipoid P per 100 ml.

Concentration of Electrocytes in the Plasma and Tissue Fluid

Each mineral constituent of the plasma and tissue fluid was estimated separately. Owing to the practical difficulty of estimating the various organic acids (hydroxy, ketonic, and oxyproteinic) the concentration of total organic acids was calculated from the difference between the total kation and the anion plus protein concentration of the fluids. In a small number of cases the lactic acid content of the plasma and tissue fluid was estimated. The results in normal pregnancy and in the four groups of pre-eclamptic oedema are shown in Table V. Before discussing these figures it is necessary to consider the manner in which osmotic equilibrium between

TABLE V

ELECTROLYTES IN THE PLASMA AND TISSUE FLUID IN NORMAL PREGNANCY AND DIFFERENT DEGREES OF PRE-ECLAMPTIC OEDEMA

	Normal Pregnancy (6 Cases)		Pre-eclamptic Oedema (45 Cases)							
			Group 1 (6 Cases)		Group 2 (11 Cases)		Group 3 (12 Cases)		Group 4 (16 Cases)	
	Plasma	Tissue Fluid	Plasma	Tissue Fluid	Plasma	Tissue Fluid	Plasma	Tissue Fluid	Plasma	Tissue Fluid
Na′ ..	130.0±1.93*†	124.4±1.97	130.5±1.47	127.8±1.58	131.9±1.11	132.9±1.72	133.1±1.60	135.9±1.87	134.9±1.17	139.9±1.17
	126.0–134.0	120.7–127.0	128.0–134.8	124.8–130.7	127.8–136.0	128.4–138.4	129.7–136.6	131.8–139.2	132.0–139.1	134.8–144.4
K′	5.1±0.37	3.9±0.46	5.0±0.25	3.7±0.25	4.9±0.18	3.8±0.26	5.0±0.28	3.8±0.23	4.7±0.42	3.6±0.41
	4.3–6.0	3.4–4.4	4.4–5.7	3.2–4.4	4.3–5.2	3.2–4.9	4.3–5.5	3.0–4.2	3.7–5.4	3.2–4.0
Ca″ ..	5.3±0.57	3.8±0.48	5.2±0.32	4.0±0.59	5.1±0.20	3.9±0.26	5.1±0.29	3.6±0.22	4.9±0.50	3.5±0.19
	4.5–5.8	3.3–4.3	4.6–5.8	3.6–4.4	4.5–5.4	3.2–4.6	4.5–5.4	3.2–4.1	3.3–5.5	3.2–4.0
Mg″ ..	2.3±0.16	2.2±0.20	2.2±0.13	2.0±0.13	2.3±0.09	2.0±0.14	2.2±0.12	2.2±0.12	2.1±0.16	2.0±0.24
	2.0–2.5	1.8–2.7	1.9–2.5	1.7–2.3	2.1–2.6	1.6–2.3	1.8–2.4	1.8–2.4	1.8–2.7	1.3–2.8
Total base	142.7±1.84	134.3±1.10	142.9±1.47	137.5±1.08	144.2±2.60	142.6±2.30	145.4±1.67	145.5±1.61	146.6±1.88	148.8±1.63
	139.1–147.0	130.6–136.7	140.2–147.3	135.2–142.2	138.3–146.9	137.8–147.6	140.4–148.9	141.0–148.5	143.6–152.9	144.0–152.0
Cl′ ..	100.2±1.69	106.4±2.22	101.4±1.0	107.1±1.11	102.4±1.51	108.7±1.58	102.5±1.16	108.9±1.90	103.4±0.97	109.8±2.14
	97.0–104.5	102.5–111.0	99.0–103.6	104.6–109.0	98.7–106.4	104.9–112.0	98.7–105.0	103.2–112.5	100.0–105.1	102.8–115.9
HCO3′	22.6±0.71	23.9±1.23	22.1±0.58	22.2±0.57	22.0±0.57	21.8±0.52	21.5±0.57	20.8±0.56	20.6±0.47	20.0±0.50
	21.8–23.5	21.8–24.5	20.4–23.2	20.8–23.0	20.4–23.8	19.8–22.9	20.4–23.0	19.7–22.0	19.5–22.0	18.7–21.4
HPO4″	2.0±0.26	1.5±0.23	2.5±0.24	3.0±0.26	2.9±0.16	3.4±0.30	3.2±0.28	4.0±0.21	4.3±0.33	5.5±0.30
	1.5–2.4	1.2–1.8	2.0–2.9	2.5–3.6	2.4–3.2	2.6–4.0	2.7–3.9	3.4–4.7	3.5–5.3	4.8–6.2
SO4″	0.7±0.16	0.5±0.16	0.8±0.09	0.8±0.11	0.8±0.09	1.0±0.17	1.0±0.18	1.0±0.12	0.9±0.15	1.0±0.13
	0.5–1.1	0.3–0.7	0.6–1.0	0.7–0.9	0.6–1.0	0.6–1.5	0.6–1.4	0.6–1.2	0.6–1.2	0.6–1.2
Prot.	15.6±0.64	0.5	13.7±0.23	0.5	13.1±0.21	0.5	13.2±0.85	0.5	12.6±0.34	0.5
	15.1–15.9	—	13.0–14.1	—	12.6–13.6	—	12.6–14.0	—	12.0–13.5	—
Org. A.	1.6±0.62	1.5±0.38	2.1±1.16	3.9±0.76	3.0±1.14	7.2±0.87	4.0±0.54	10.3±1.54	4.8±0.94	12.0±1.0
	0.7–3.4	1.0–2.2	0.8–5.2	2.3–4.8	0.5–5.7	3.4–9.8	2.3–5.2	5.7–13.3	3.8–8.6	9.7–14.8

* All values represent the concentration in mEq. per litre of plasma and tissue fluid.

† The above figures in each case represent the average together with the probable error and the minimal and maximal values.

plasma and tissue fluid is achieved. When equilibrium is established the levels which the various ingredients attain are not related to their absolute concentrations by volume in the two media, but to the weight of the water in which they are dissolved. This is of little significance when dealing with freely diffusible solutes, but it is of some consequence when comparing plasma and tissue fluid. The large molecules of protein displace a considerable amount of the water in which they are dissolved. A litre of plasma containing 7% protein holds only 935 g. of water. The plasma water estimated in four normal cases was found to vary between 937 and 944 with an average of 940 g. per 100 g. of plasma. The water content of tissue fluid (average of six cases) was 980 g. Plasma contains approximately 16 milli-equivalents of protein whereas the tissue fluid only holds about 0.5 Thus, if the concentrations of the solutes are expressed in terms of weight per volume of plasma and tissue fluid the results may suggest that these fluids do not obey Donnan's law of equilibrium. It can therefore be understood that the marked difference in protein concentration on either side of the capillary membrane makes a readjustment of the electrolyte levels in the two fluids necessary. At the *p*H of plasma the proteins act as anions and therefore a diminished concentration of the total base can be expected. Thus the concentration of sodium, potassium, and calcium in the normal tissue fluid is found to be less than that of the plasma. The extremely low protein content of the tissue fluid, on the other hand, calls for a relatively higher concentration of anions to maintain equilibrium. The actual values of both kations and anions, however, do not quite agree with theoretical levels. Potassium and calcium form physiologically inert compounds with proteins, and the anions are affected by the constant flow of carbonic acid from the tissues to the plasma and the resulting chloride shift. These factors are not of themselves important, and, provided that the variation in the percentage of water in the two fluids is taken into consideration, it is found that plasma and tissue fluid obey Donnan's law.

Analysis of the data indicates the similarity between the electrolyte pattern of the plasma and that of tissue fluid in normal pregnancy. The total base concentration of the plasma is to some extent less than that in non-pregnant subjects, a finding which is in accord with that of other investigators (Dieckmann, 1942). This is probably caused by the decrease in the concentration of plasma proteins resulting in a relative increase of plasma water. On the basis of the figures given above for the water content of plasma and tissue fluid it would appear that the electrolyte partition between these two fluids closely approximates the theoretical values. B_P/B_T* is 1.108 and A_T/A_P is 1.010. The slight excess of base in the plasma is related to the higher concentration of protein. The difference in the base concentration between these two fluids is 14.8 milli-equivalents per 1,000 g. of water, which almost corresponds to the milli-equivalent value of the plasma proteins. These findings for the plasma-tissue fluid ratios of base and acid agree with those observed by Greene *et al.* (1931) in experimental dialysates. The concentration of the anions in the tissue fluid is almost identical with that of the plasma. Phosphates, sulphates, and organic acids are slightly reduced in the tissue fluid. Chlorides are slightly in excess, and carbonates are practically equally distributed on either side of the

*B_P, B_T, A_P, A_T represent concentrations respectively of total base and total acid per litre of serum water or tissue fluid water.

TABLE VI

TISSUE FLUID RATIO OF ELECTROLYTES IN PLASMA IN NORMAL PREGNANCY AND PRE-ECLAMPTIC OEDEMA

	Na′	K′	$\sqrt{Ca}''$	$\sqrt{Mg}''$	ΣB	Cl′	HCO_3'	$\sqrt{HPO_4}''$	$\sqrt{SO_4}''$	Or.A	ΣA.
Normal pregnancy (6 cases)	1.061	1.130	1.197	1.036	1.153	0.980	0.098	1.179	1.173	1.111	0.996
Pre-eclampsia: Group 4 (16 cases) ..	0.992	1.325	1.189	1.035	1.012	0.971	1.054	0.900	1.971	2.400	0.929

capillary membrane. The excess of chloride is more apparent than real. The true difference is only 2 milli-equivalents per 1,000 g. of water. The ratio of the anions in the plasma and tissue fluid is shown in Table VI. It is obvious from these figures that the nature of the tissue fluid in normal pregnancy does not vary from that of the non-pregnant subject.

The composition of the plasma and tissue fluid scarcely varied from normal in minor degrees of pre-eclamptic oedema. If, however, the figures for the various groups are examined certain changes become obvious. These are considered under their respective headings.

Increase of Plasma Electrolytes.—There is a slight rise in the concentration of the plasma electrolytes. This is not due to haemo-concentration, for, with the fall in proteins, the water content of the plasma actually increases. In four cases of generalized oedema we found the plasma water to vary between 949 g. and 960 g. with an average of 954 g. per litre. However, the actual increase in electrolytes is only 2 milli-equivalents per 1,000 g. of water. Examination of the individual constituents shows that there is a progressive increase of plasma sodium as the oedema becomes worse. In severe cases it is raised by 3.1 milli-equivalents per 1,000 g. of plasma water, and it is accompanied by a decrease in the concentration of other kations to the extent of just over 1 milli-equivalent. In view of the highly active nature of sodium ions these changes are probably of a compensatory nature.

On the acid side the change principally affects the organic acids and inorganic phosphates. The concentration of the former in severe cases is raised by 3.2 milli-equivalents or 200% and that of the latter by 2.3 milli-equivalents or 115% above the level in normal pregnant subjects. There is little change in the true concentration of the chlorides. The same holds good for sulphates, but the bicarbonates decrease by 3.3 milli-equivalents per 1,000 g. of plasma water or 13.3% of the normal value. There is thus a slight positive balance of plasma anions in cases of oedema. This is shown by the kation : anion ratio. In normal pregnancy this is 1.123 and in cases of generalized oedema it is 1.094.

Increase of Tissue Fluid Electrolytes.—The increase in electrolytes is more marked in the tissue fluid than in the plasma. The total increase amounts to 14.5 milli-equivalents per 1,000 g. of tissue water (compared with 2 milli-equivalents per 1,000 g. of plasma water). This is 7.25 times the corresponding increase in the plasma. The disproportionate rise in tissue fluid electrolytes is contrary to the

ordinary mechanism of tissue fluid formation and indicates that the retention of fluid in the tissue spaces is the result of some active process which upsets the normal equilibrium existing on either side of the capillary membrane. The qualitative changes, however, run parallel with those in the plasma, and the increase in electrolytes is brought about by changes in the sodium, phosphates, and organic acids. The concentration of sodium rises by 25.3 milli-equivalents or 20.2% of the normal level. As in the plasma this is accompanied by a slight fall in the other kations. The organic acids, phosphates, and chlorides all show an increase. The rise in chlorides, however, is only 3.4 milli-equivalents, which is almost the same as the increase in the plasma. The partition of chloride in plasma and tissue fluid in oedematous patients thus remains the same as in normal pregnancy. The organic acids are raised by more than 10 milli-equivalents or 700% and the phosphates by 4 milli-equivalents per 1,000 g. water or 266% of the normal. There is a fall in bicarbonate by 3.9 milli-equivalents.

Partition of Electrolytes between Plasma and Tissue Fluids.—In normal pregnancy the partition of electrolytes between plasma and tissue fluid is in fair agreement with the formula $B_P/B_T = A_T/A_P$. In toxaemia, however, these ratios are disturbed, and with marked oedema. B_P/B_T is 1.012, but A_T/A_P is 1.076. This indicates that in these cases there is a retention of electrolytes in the tissues. As can be seen from Table VI the retention is mainly due to an increase in sodium, organic acids, and phosphates, and the sodium is apparently combined with these anions. Theoretically the ratio of plasma phosphates to tissue fluid phosphates should be less than unity, but it is slightly more, both in normal pregnancy and in experimental dialysates. This is usually explained on the assumption that the phosphates of the plasma exist partly in a non-diffusible form. In toxaemia the position is reversed and the non-diffusible tissue fluid phosphates are obviously increased. According to Greenberg, Larson, and Tufts (1935) and Grollman (1927) when the plasma phosphates rise above a certain level it forms non-diffusible salts with calcium. The reduced concentration of calcium in the tissue fluid makes such an explanation impossible in cases of toxaemia of pregnancy, and it seems more likely that the phosphates exist in complex combination with organic substances.

Discussion

The physical factors and variations in capillary permeability can play only a minor role in the causation of oedema in pre-eclamptic toxaemia. It is possible that these factors increase the severity of oedema, but they play little or no part in the initiation and maintenance of the process. It has been pointed out that in these cases there is a progressive fall in plasma proteins, and this of itself by its effect on osmotic pressure may tend to cause oedema. This change, however, cannot be responsible for the progressive accumulation of fluid in the tissue spaces. A continual outflow of fluid from the plasma would soon restore the normal level of colloid osmotic pressure and conditions would tend to return to normal. The critical level of colloid osmotic pressure of the plasma in relation to oedema is 20 cm. of water. Dieckmann (1942) observed that the oncotic pressure in 15 cases of normal pregnancy was 28.7 cm. of water, and in 14 cases of toxaemia the figure was 24.9 cm. of water. We have studied the oncotic pressure in 45 cases of toxaemia and in only

11 was the value below the critical level. In addition, these 11 cases included patients with varying degrees of oedema, some of them minor. Visible oedema was also noted in six cases in which the colloid osmotic pressure of the plasma was above 24 cm. of water.

None of the other organic constituents, with the exception of organic acids and phosphates, appear to bear any relation to the onset and progress of toxaemic oedema of pregnancy. There would, however, appear to be justification for suggesting that the process is associated with the changes in the concentration of sodium, phosphorus, and organic acids. We have not found chloride to play any part other than a passive one in the mechanism. It seems reasonable to suggest that the sodium is retained in the tissues to neutralize the excess of anions. Whether the retention of sodium is an active process due to hyperactivity of the adrenal cortex cannot at present be decided. Our own studies tend to indicate that if such a mechanism is at work it is of a secondary nature.

We would suggest that the primary factor in the oedema of toxaemia of pregnancy is a disorder of metabolism, probably related to carbohydrate, resulting in an excess of organic acids and phosphates in the tissue fluids. Why these substances are retained is difficult to say, and an answer awaits further investigation.

Summary

A comparative study of the relative concentration of electrolytes in the plasma and tissue fluid in six cases of normal pregnancy and 45 cases of pre-eclamptic oedema showed that in normal pregnancy the electrolytic pattern on either side of the capillary membrane is the same as that in non-pregnant persons and in experimental conditions, although the total concentration of electrolytes in these regions in pregnancy is slightly less than that in the non-gravid state.

In pre-eclampsia there is an increased concentration of electrolytes in the tissue fluid. This change progresses as the oedema increases in severity. The raised level of electrolytes in the tissue fluid is due to an active retention of sodium in the tissue spaces, associated with a corresponding retention of organic acids and inorganic phosphates in this region. There is no evidence of retention of chlorides in the tissue fluid in pre-eclamptic oedema. It is suggested that the increased concentration of organic acids and phosphates is related to an upset in tissue carbohydrate metabolism.

References

Albano, G. (1927). *Folia Gynaec.*, **24**, 81.
Boyd, E. M. (1935). *Amer. J. Obstet Gynec.*, **30**, 323.
Chesley, L. C., and Chesley, E. R. (1943). *Amer. J. Obstet. Gynec.*, **45**, 748.
Dieckmann, W. J. (1942). *Toxemias of Pregnancy*. London.
Greenberg, D. M., Larson, C. E., and Tufts, E. V. (1935). *Proc. Soc. exp. Biol., N.Y.*, **32**, 647.
Greene, C. H., Bollman, J. L., Keith, N. M., and Wakefield, E. G. (1931). *J. biol. Chem.*, **91**, 203.
Grollman, A. (1927). Ibid., **72**, 565.
Hastings, A. B., Salvesen, H. A., Sendroy, J., and Van Slyke, D. D. (1927). *J. gen. Physiol.*, **8**, 701.
Hellmuth, K. (1922). *Zbl. Gynäk.*, **46**, 290.
King, E. J. (1946). *Micro-Analysis in Medical Biochemistry*. London.
Loeb, L. (1923). *Oedema*. Baltimore
Mukherjee, C., and Govan, A. D. T. (1950). In the press.
Peters, J. P., and Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, vol. 2. London.
Taylor, H. C., Warner, R. C., and Welsh, C. A. (1939). *Amer. J. Obstet Gynec.*, **38**, 748.

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TECHNICAL METHODS

A SIMPLE VISUAL TURBIDIMETRIC ESTIMATION OF SERUM GAMMA GLOBULIN

BY

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It was shown in our paper (page 260) comparing electrophoresis and precipitation methods that the γ-globulin fraction of serum could be determined by precipitation with a saline ammonium sulphate reagent giving results which were in good agreement with those obtained by electrophoresis. It was noticed in these precipitations that the turbidity produced by the addition of the reagent appeared to be directly proportional to the amount of γ-globulin, as determined by subsequent micro-Kjeldahl analysis. Since this might provide a simple method of estimating this fraction, it was decided to investigate further the correlation between serum γ-globulin and the turbidity produced.

Method

Serum, 0.1 ml., is added to 4.9 ml. of saline ammonium sulphate solution (prepared by dissolving 193 g. A.R. ammonium sulphate and 40 g. sodium chloride in distilled water and making up to 1 litre) in a test tube, which is inverted several times to mix, and allowed to stand for 15 minutes to develop maximum turbidity. The turbidity is then read visually against the turbidity standards of Kingsbury, as in the thymol turbidity test, the units of turbidity being calculated in the same way. Where the turbidity is high, the mixture is diluted by adding a further 5, 10, or 15 ml. of reagent and the reading obtained multiplied by the corresponding dilution factor. These proportions are slightly different from those used in the chemical estimation since, if the latter are used, the turbidity produced is outside the range of the standards.

Alternatively a fresh volume of 0.1 ml. of serum may be added to 9.9 ml., 14.9 ml., or 19.9 ml. of reagent, and the reading obtained multiplied by the appropriate dilution factor.

γ-Globulin was estimated chemically by the saline ammonium sulphate method.

Results.—The γ-globulin and the γ-globulin turbidity were determined in 70 sera, mostly from children under the age of 14 years, including normal children and others suffering from a variety of conditions. In Fig. 1 are shown the chemical and turbidity determinations plotted against each other. There is a linear relation, the results falling on or near a

line which cuts the axis at a point equivalent to 0.21 g. per 100 ml. From the results. the following formula was calculated:

γ-Globulin (g. per 100 ml.) = 0.08 U. + 0.21 where U = units of γ-globulin turbidity.

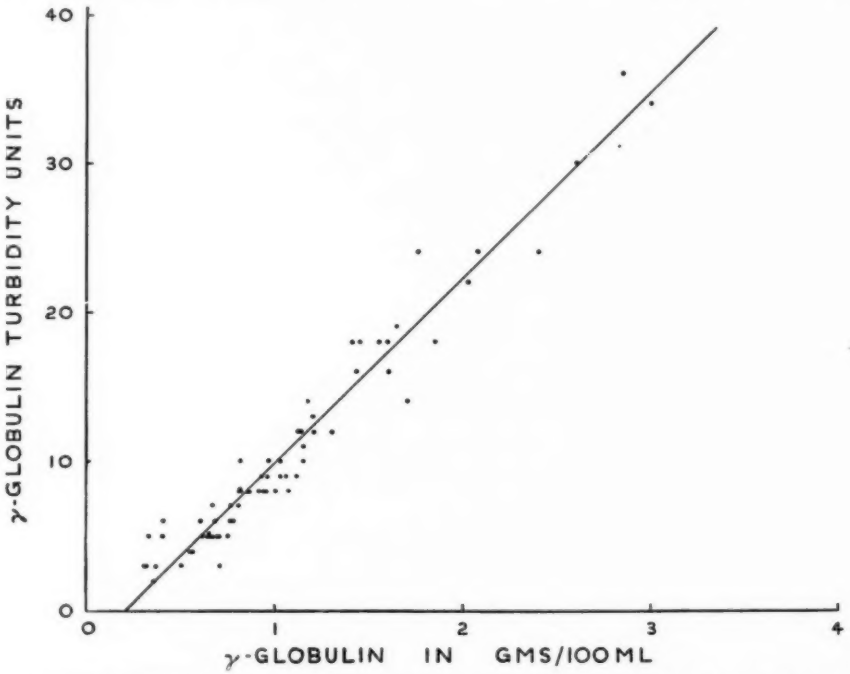

FIG. 1.—On this graph one very high result (80 units—6.72 g. per 100 ml.) has not been shown although it fell close to the line.

Discussion

Kunkel (1947) has described a similar method for the turbidimetric estimation of γ-globulin, although the precipitating reagent is of a different type. It is not claimed that the method described above is as accurate as the micro-Kjeldahl estimation of the globulin precipitate. It has the great merit of ease and simplicity, requiring only common reagents and apparatus. No special correction was needed for lipaemic sera, since lipaemia did not appear to alter the γ-globulin turbidity. The standard deviation of the two methods over the whole range of results is ± 0.13 g. per 100 ml., and the average percentage difference is 11.6. For results under 1.2 g. per 100 ml., the standard deviation is ± 0.11 g. per 100 ml. and the average percentage difference is 12.3, whereas for results above 1.2 g. per 100 ml., although the standard deviation is ± 0.19 g. per 100 ml., the average percentage difference is 9.0.

The method should be useful not only as a liver function test as Kunkel, Ahrens, and Eisenmonger (1948) have shown, but also in following up changes in γ-globulin in such conditions as nephritis and rheumatic fever. It is useful also as a method for the rapid survey of γ-globulin levels in large groups, and we are at the moment engaged in determining normal levels in infants and children.

Whilst this work was in progress, a similar method has been described by Popper and de la Huerga (1950), who, however, use a spectrophotometer or photoelectric colorimeter for measuring turbidities. We do not think this is necessary, since the accuracy of the method is not great enough to warrant it. If greater accuracy is required, it is necessary to carry out a micro-Kjeldahl analysis.

References

Kunkel, H. G. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 217.
—— Ahrens, E. H., and Eisenmonger, W. J. (1948). *Gastroenterologia, Basel*, **11**, 499.
Popper, H., and de la Huerga, J. (1950). *J. Lab. clin. Med.*, **35**, 459.

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THE GRAVIMETRIC DETERMINATION OF PREGNANEDIOL IN URINE

BY

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The methods of Venning and Browne (1937) and Astwood and Jones (1941) form the basis of most of the methods that have been described for the determination of pregnanediol in human urine. In the former, the pregnanediol is separated as the glucuronidate, the amount being determined gravimetrically. In the latter, the urine is subjected to acid hydrolysis and the amount of free pregnanediol determined gravimetrically. There have been a number of criticisms of the Venning-Browne method. The chief is that the isolated pregnanediol glucuronidate may be contaminated with a 20-ketosteroid. In a recent study, I (Tompsett, 1949 ; and unpublished data) found that, although 20-ketosteroids exist in appreciable quantities in the urine of late pregnancy, they are not found in appreciable quantities in the urine of non-pregnant subjects or in early pregnancy, except possibly in cases of adrenocortical tumour.

Quantitative methods have been described for the determination of pregnanediol in quantities of urine less than the 24-hour output (Sommerville, Marrian, and Kellar, 1948 ; Henderson, Maclagan, Wheatley, and Wilkinson, 1949). The amount of pregnanediol is determined colorimetrically and this necessitates the provision of pregnanediol as a standard. In this laboratory, because there has never been any difficulty in obtaining such, complete 24-hour collections have always been used. A modified Astwood and Jones (1941) method is employed. No standard substance is required and the actual time of weighing is small if one employs a modern balance. Although it takes two and a half days to obtain a result, we have found that the process can be broken up into a number of convenient stages which fit into the general routine of the laboratory. In an extensive investigation, it has been found that the excretion of pregnanediol by the male is less than 1 mg. per day, and in the female during the reproductive phase of life the maximum is 4.5–6.5 mg. per day. In late pregnancy it has been found that pregnanediol as determined by this method agrees very closely with the content of non-ketonic alcohols of the neutral steroid fraction (Tompsett, 1949).

Materials and Method

Reagents.—The following reagents are required :

1. Redistilled toluol.
2. Concentrated hydrochloric acid.
3. Aqueous 10% sodium hydroxide.
4. Aqueous 0.1N. sodium hydroxide.
5. Absolute alcohol (redistilled).

Apparatus.—The apparatus required is:

1. Two-litre flasks fitted with reflux condensers (all-glass).
2. Vacuum distillation apparatus (all-glass) fitted with 500-ml. flasks.
3. 100-ml. conical flasks.
4. Two-litre separating funnels.
5. Seven-cm. No. 42 Whatman filter papers.

The entire 24-hour collection of urine is introduced into a 2-litre flask fitted with a reflux condenser together with 1/10 of its volume of concentrated hydrochloric acid and 200 ml. toluol. The mixture is heated to boiling, the boiling continued for 15 minutes, and then cooled rapidly in running water.

The mixture is transferred to a 2-litre separating funnel. The toluol is separated and the aqueous fraction extracted with two further quantities of 200 ml. toluol. The combined toluol extracts are washed twice with two 100 ml. quantities of 10% sodium hydroxide and twice with 100 ml. quantities of water. The toluol extract is evaporated down to dryness in a 500 ml. flask attached to a vacuum still. It is essential to have the flask heated by a water bath and to begin heating from the cold, otherwise trouble from "bumping" may be encountered.

The dry residue is dissolved in 10 ml. absolute alcohol, the solution being transferred to a 100 ml. conical flask, and 40 ml. hot (about 70° C.) 0.1N. sodium hydroxide solution added to the alcoholic solution. (This process can be used to transfer completely the contents of the 500 ml. flask into the 100 ml. conical flask.) The mixture is allowed to cool to room temperature after which the flask is placed in a refrigerator (5° C.) overnight. The contents of the flask are filtered through a 7 cm. No. 42 Whatman filter paper. The residue is washed with 40 ml. cold water (5° C.). (The cold water should be transferred from the original conical flask.) The filter paper containing the residue is put back into the original conical flask and 10 ml. absolute alcohol added. When solution of pregnanediol has been effected, 40 ml. of hot water (about 70° C.) is added. The mixture is allowed to cool to room temperature after which it is placed in a refrigerator (5° C.) overnight. The contents of the flask are then filtered through a 7 cm. No. 42 Whatman filter paper. The residue is washed with 40 ml. cold water (5° C.). The residue on the filter paper is dissolved in about 40 ml. absolute alcohol (contained in the original conical flask), the filtrate being collected in a weighed 50 ml. beaker. The alcohol is evaporated off in a hot air oven maintained at 100° C. The dry beaker is allowed to cool in a desiccator. The increase in weight represents pregnanediol separated.

Summary

The determination of pregnanediol in urine by a gravimetric method is described.

References

Astwood, E. B., and Jones, G. E. S. (1941). *J. biol. Chem.*, **137**, 397.
Henderson, J., Maclagan, N. F., Wheatley, V. R., and Wilkinson, J. H. (1949). *J. Endocrinol.*, **6**, 41.
Sommerville, I. F., Marrian, G. F., and Kellar, R. J. (1948). *Lancet*, **2**, 89.
Tompsett, S. L. (1949). *J. Endocrinol.*, **6**, 192.
Venning, E. H., and Browne, J. S. L. (1937). *Endocrinology*, **21**, 711.

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A SIMPLE ESTIMATION OF SALICYLATE IN SERUM

BY

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The control of salicylate therapy by frequent estimation of the blood level requires a simple method suitable for use in a routine clinical laboratory. The method of Brodie, Udenfriend, and Coburn (1944) is generally accepted on account of its accuracy but is somewhat too laborious for a large number of estimations. A number of other methods are known but none has found general acceptance. Some of these were given preliminary trials in the summer of 1948. They were the methods of Camponovo (1945, 1946), Barker (1936), Volterra and Jacobs (1947), Weichselbaum and Shapiro (1945), Coburn (1943), Mallick and Rehmann (1945), and Vélez-Orozco and Guerra (1946). Camponovo's (1945, 1946) method seemed the only satisfactory procedure. At the time this was available to us only in its abstracted form, and restandardization was necessary. A gradually evolved modified version has been in use in this laboratory for over a year. Comparing this with Camponovo's full papers shows a considerable difference in technique, our version being equally accurate but simpler.

Experimental

Principle.—Serum is added to acetone to precipitate proteins, dissolve salicylate without previous acidification and, possibly, free any salicylate held by protein. The drug is transferred to an aqueous-acetone phase by the addition of water and extraction with a second organic solvent. The salicylate content of this phase is determined by the well-known colour reaction with ferric chloride.

Reagents.—1. Acetone A.R.
2. Acetone, 15% (v/v) in water.
3. Methylene chloride (laboratory reagent quality).
4. Ferric chloride, stock solution, 20% (w/v) in water.
5. Ferric chloride, working solution, 0.05% (w/v). The working solution is made by diluting 0.25 ml. 4 to 100 ml. with water. When this reagent is read in the photoelectric colorimeter under the same conditions as a test solution, the reading should not exceed one division.

Method.—Serum (1.0 ml.) is added slowly to 5.0 ml. acetone, preferably contained in a 4-in. "quickfit" test-tube fitted with a ground glass stopper. The tube is shaken vigorously for three minutes, allowed to stand for 20 minutes, and centrifuged at moderate speed for five minutes.

Clear supernatant liquid (4.0 ml.) is added to a centrifuge tube containing 1.0 ml. methylene chloride and 3.0 ml. 0.05% ferric chloride. The tube is shaken with inversion for one minute and centrifuged for five minutes. The purple upper layer is removed by means of a Pasteur pipette and compared with a standard solution, using

a photoelectric colorimeter with medium size colorimeter tubes (1 cm. diam.), an Ilford 624 or 404 green filter, and setting the instrument to zero with a water blank. A Dubosq-type visual colorimeter can be used with standard 2. The colours may be read immediately and are stable for at least two hours. Too deeply coloured solutions are diluted with 15% acetone before reading.

Standards.—(1) *Stock Standard Solution* (200 mg. salicylic acid per 100 ml.).—Sodium salicylate, 579.7 mg., is dissolved in 250 ml. water.

(2) *Dilute Standard Solutions* (20 and 40 mg. per 100 ml.).—These are prepared by diluting (1) accordingly. The dilute standard, 1.0 ml., is then treated in the same way as serum. This standard is suitable for use both with visual and photoelectric colorimeters.

(3) *Simplified Dilute Standard Solution* (for use with photoelectric colorimeter only).—This is prepared by diluting 3.6 ml. of stock standard to 100 ml. with water; 4.0 ml. of this mixed with 2.0 ml. 0.05% ferric chloride gives a solution equal in colour intensity (though not in colour) to that of a 40 mg. per 100 ml. standard solution.

All three solutions are preserved with chloroform and are stable for several months.

Results

The present method cannot be applied to whole blood. This is contrary to Camponovo, who describes his procedure as suitable for use with serum, plasma, or whole blood. Oxalated plasma is also unsuitable owing to the colour reaction between oxalate and ferric chloride. The use of the two solvents, however, permits of the analysis of haemolysed, pigmented, and lipoid sera. Camponovo records a yellowish tinge in the aqueous acetone extracts of some sera. This is overcome by adding 0.1 ml. saturated calcium chloride solution to the acetone used in the first extraction. In our experience this has not been necessary. No blank readings have been found with sera not containing salicylate.

Using Ilford 404 or, preferably, 624 filters, the colour intensities are proportional to concentration in the range between 5 and 100 mg. salicylic acid per 100 ml.

The colour obtained varies with the relative proportions of water and acetone in the medium. Solutions containing more water are bluer; those containing more acetone, browner. In the conditions of the determination, methylene chloride reduces the volume of the coloured layer by 24%, the removal of acetone giving a deeper and bluer solution. In both these respects methylene chloride is preferable to the chloroform used by Camponovo. Other solvents have been tried; these are carbon tetrachloride, ethylene chloride, benzene, toluene, xylene, 2-octanol, and ethyl acetate. Although the colour of the final aqueous solutions varies with these solvents, the colour intensity in most cases depends simply on the degree of concentration

TABLE

RECOVERIES OF SALICYLATE (STOCK STANDARD SOLUTION) ADDED TO SERUM

Serum Salicylic Acid Level (mg./100 ml.)	No. of Experiments	Recovery (%)	
		Range	Mean
20	33	87–92	90
40	13	91–96	94

achieved by the extraction. Benzene seems a suitable second solvent, provided the colorimeter tubes can be used for the second extraction and for centrifuging; the colours could then be read without separating the two layers.

Recoveries from serum are shown in the Table.

Summary

A method for estimating salicylate in serum has been evolved from Camponovo's (1945, 1946) double extraction procedure. The method is simple and well adapted for routine use. It requires serum (which may be haemolysed, pigmented, or lipoid) and should not be used with oxalated plasma or with whole blood.

REFERENCES

Barker, M. H. (1936). *J. Amer. med. Ass.*, **106**, 762. Compare Norris, R. F., Martens, V. E., and Sunderman, F. W. (1945). *Amer. J. clin. Path.*, **15**, 196.
Brodie, B. B., Udenfriend, S., and Coburn, A. F. (1944). *J. Pharmacol.*, **80**, 114.
Camponovo, P. B. (1945). *Rev. Soc. argent. Biol.*, **21**, 309.
—— (1946). *Rev. Soc. bioquim. argent.*, **13**, 67.
Coburn, A. F. (1943). *Bull. Johns Hopk. Hosp.*, **73**, 435.
Mallick, S. M. K., and Rehmann, A. (1945). *Ann. Biochem. exp. Med.*, **5**, 97.
Vélez Orozco, F., and Guerra, F. (1946). *Arch. Inst. Cardiol. Mex.*, **16**, 131. (Abstract in *Chem. Abstr.* (1946), **40**, 7261[8].)
Volterra, M., and Jacobs, M. D. (1947). *J. Lab. clin. Med.*, **32**, 1282.
Weichselbaum, T. E., and Shapiro, I. (1945). *Amer. J. clin. Path. Tech. Sect.*, **9**, 42.

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REVIEWS

An Introduction to Pathology. By G. Payling Wright. 1950. London : Longmans, Green. Illustrated. Pp. 569. Price 30s.

There has been a serious need for such a book as this for the last quarter of a century, and it is most gratifying to discover that it has at last been written by an author with so wide a knowledge of the scientific aspect of pathology and so outstanding a talent for accurate generalization. It is planned to give the student an adequate understanding of the basic mechanisms which underlie disease processes. It deals with the principles of pathology in terms of the more fundamental sciences of biology and physiology. Every opportunity is taken to put life into the static facts of morbid anatomy and histology by blending them with the discoveries of modern biology, physiology, experimental pathology, and immunology. Pathogenesis is heavily emphasized and the story of pathology is told in lucid and distinguished English against the background of time. So skilfully has this been done that Professor Payling Wright will undoubtedly earn the gratitude, not only of the junior clinical student for whom the book was written, but also of those of us who undertake the difficult task of teaching general pathology.

The introduction is an impressive piece of writing. It consists for the most part of an account of the history of pathology in terms of the evolution of science and has the same dynamic quality as the rest of the book. Other outstanding chapters are those dealing with the body's defences against infection, the inheritance of abnormalities, haemorrhage, thrombosis, and the aetiology of tumours. The references, which cover an uncommonly wide field, are wisely selected, and of the 250 well-chosen illustrations more than half are photomicrographs and all are of high standard.

Professor Payling Wright's book has several qualities which will ensure its success. It is remarkably free from prejudice and dogmatism ; it is easy to read, well balanced, and small in size, but above all it is inspiring.

GEOFFREY HADFIELD.

Disorders of the Blood. By Sir Lionel Whitby and C. J. C. Britton. 1950. London: J. and A. Churchill, Ltd. Sixth edition. Illustrated. Pp. 759. Price 42s.

The appearance of the new "Whitby and Britton" in its sixth edition will be widely welcomed. It has been considerably altered in many respects, and includes an extensive revision of certain chapters to include much newer work on pigment metabolism, the mechanics of coagulation, and haemagglutination. It is only four years since the last edition, but these years have been filled with a vast and growing literature in both the clinical and laboratory aspects of haematology. Such new advances as the treatment of pernicious anaemia with B_{12}, intravenous iron therapy, folic acid metabolism, and the use of tracer elements are all of vital importance to the haematologist. The detailed study of blood groups and subgroups has also provided greatly increased knowledge in a far from stabilized field of investigation.

It is difficult when bringing a textbook up to date to know exactly which new work to include as acceptable, and which fairly to omit, and withal to keep a balanced view of the newer pathology. The authors have managed remarkably well to present in the revised chapters such a balance. The new additions on the absorption and excretion of iron give a much clearer view of the method by which the iron radicle is utilized and of its storage and metabolism, and the diagram illustrating the processes involved is more than useful. The chapter on pernicious anaemia is particularly helpful, and presents an up-to-date picture of the pathology and treatment of the condition. The information on blood coagulation and the haemorrhagic diseases is well arranged, and Macfarlane's table on the factors involved in coagulation enables the reader to follow the discussion with ease.

Nearly 100 pages at the end of the book are devoted to a description of technical methods, and here again some revision has improved a most useful guide for the laboratory worker.

It is most encouraging to the editors of this journal to see such an extensive use made of the material published in it during the past two years, with references to many of the recent papers published here. The references throughout are very adequate, and yet they never intrude upon the reader. Some of the new plates are excellent, especially those of the bone marrow where reference to the key identifying the individual cell is immediately accessible. Other new plates are, however, not of the standard which we expect in this textbook. The new colour plates 6, 9, and 11 (i) show entirely different colour from the older plates, and are not an accurate representation of the normally used staining methods. Comparing plate 5 (p. 45) with its counterpart, plate 3, in the previous edition, neither colour nor details, particularly of the reticulocyte, compare favourably with the earlier illustrations. (It is possible that this occurred only in the reviewer's copy.) It is also rather disturbing to find evidence of uncertain sub-editing in the use of those abbreviations which have become standard in scientific literature. It is to be hoped that reprints or any new editions will remove the indiscriminate use of the abbreviations " g," " gm," " mg," and " mgm." This is particularly striking in some pages—e.g., on page 138 where line two refers to " 3.34 mg. of iron," and, thereafter, in consecutive lines refers to " 26 mgm. of iron." Similarly page 635 refers to " 0.5 gram " and " 0.15 gram," and a little further down uses " 25 g. of Na_2HPO_4," whereas two pages later, in the method for peroxidase staining, we are told to " rub 0.2 gm. of benzidine." These minor irritations could be removed by a little careful sub-editing.

Only one criticism remains, which it is hoped the authors will face in their next edition, and that is the vexed question of the colour index. It seems a pity to perpetuate this measurement in the light of many of the remarks made in this and other publications, which leads to the conflicting use of the term " hyperchromic anaemia " in the classification of the anaemias and in the discussion. The nomenclature is later attacked on page 58, where the term " hyperchromic " is called " misleading." As the index is based on two percentage figures, neither of which is in any way relevant, the index itself must frequently be misleading and has outlived its usefulness. There is really no more point in referring to the percentage of haemoglobin than to the percentage of urea in the blood, or the percentage of blood pressure over a stated average normal. There is no doubt that students and clinicians must be trained in the use of the new terminology, and be taught to think in terms of haemoglobin in g.%, but as long as the old method of reporting is perpetuated in Whitby and Britton, there can be no universal change-over.

It is impossible to consider in any greater detail the rest of the book. It is sufficient to say that the authors have used their judgment well in maintaining the old, and introducing new, subject matter, and that this book, already the standard " blood book " in this and many other countries, has worthily consolidated its position with this new edition.

A. GORDON SIGNY.

Practical Haematology. By J. V. Dacie, M.B., B.S.(Lond.), M.R.C.P.(Lond.). Pp. 172. Illustrated. London. J. and A. Churchill, Ltd. Price 10s. 6d.

We should like to welcome this new handbook of practical haematology, which is a product of the Department of Clinical Pathology at the British Post-Graduate Medical School in London.

It is based on practical notes for students and is an admirable laboratory book for pathologists and technicians in training.

The author makes no attempt to set down elaborate techniques, nor does he duplicate the methods, as this is essentially a practical handbook of standard methods used in a routine laboratory. The approach to the subject is logical and direct, and it is encouraging to see such a positive view taken of the value and limitation of laboratory investigations, as it is particularly the beginners in laboratory work who tend to place so much reliance on results which have an intrinsic error. This excellence is well illustrated in, for example, the short section on the red cell count (p. 36) which could with advantage be distributed from every laboratory to clinicians who tend to rely on small variations in the red cell count as a guide to progress and prognosis. Throughout the book all the methods described have been thoroughly tested, and the book will no doubt be widely used by students entering for the haematology examination of the Institute of Laboratory Technology.

The author has resisted any temptation to become too theoretical but the reason for each

step in any technique is carefully explained. The book will without doubt become a standard manual, but it will probably have to grow in size, as it becomes necessary to provide alternative methods for the more enquiring and this will undoubtedly enhance its value.

A. GORDON SIGNY.

Jordan-Burrows Textbook of Bacteriology. By W. Burrows with the collaboration of F. B. Gordon, R. J. Porter, and J. W. Moulder. 15th Ed. Philadelphia and London. W. B. Saunders. Pp. 981. Price 45s.

"Jordan and Burrows," now in its fifteenth edition with Professor W. Burrows as editor and principal author, has long been a popular textbook of bacteriology in America. Designed originally as a textbook for the undergraduate student, this present volume deals with some aspects of general bacteriology in greater detail than is required to meet the needs of our medical students. The chapter on bacterial physiology, for example, runs to 75 pages. But it should be added that the introductory chapters on bacterial variation, virulence, transmission of infection, and immunity are very readable and remarkably up to date. The chapters on individual pathogens are essentially practical; the infections with which they are associated are briefly described, the methods for laboratory diagnosis are given, and the epidemiology and control of the infection are adequately discussed. There are comprehensive chapters on medical mycology and parasitology, and Dr. F. B. Gordon contributes three chapters on filterable viruses, the virus infections of man, and bacteriophage.

Pleasant features in an American textbook are the frequent references to British work and a rather conservative attitude to new bacterial terminology. The book is beautifully produced and the illustrations and microphotographs are excellent.

ROBERT CRUICKSHANK.

ERRATA

Dr. Prunty writes: "My attention has been drawn to an error on page 104 (May, 1950). At the bottom of the section on eosinophil counts appear the words 'total count multiplied by 0.625.' This should read 'total count multiplied by $\frac{100}{64}$.'"

Dr. B. A. Thompson writes: "Further to my paper 'Dried Disc Technique for Determining Sensitivity to the Antibiotics' published in the May issue, I regret to say that I was misinformed on the name of the filter paper I used. The paper used was Postlip Mills No. 633 filter paper or Green's No. 401 filter paper."

J. clin. Path. (1950), 3, 295.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Bacterial Meningitis. Results of Treatment in Seventeen Cases with a New Sulfonamide ("Gantrisin"). RHOADS, P. S., SVEC, F. A., and ROHR, J. H. (1950). *Arch. intern. Med.*, **85**, 259.

After their previous work (*Quart. Bull. Nthwest. Univ. med. Sch.*, 1949, **23**, 104) the authors studied the effects of "gantrisin" (3:4-methyl-5-sulphanilamido-iso-oxazole) in cases of bacterial meningitis.

Ten cases of meningococcal meningitis were treated with the drug alone, two cases of meningococcal meningitis with the drug and penicillin (30,000 units three-hourly), three cases of pneumococcal meningitis with the drug and penicillin, and two cases of *Haemophilus influenzae* meningitis with the drug and streptomycin (0.1 g. intramuscularly three-hourly). The initial intravenous dose of the drug was from 2 to 6 g., with a daily maintenance dose of 6 g. (given four-hourly orally or intravenously). The average level of the free drug in the blood was 9.5 mg. per 100 ml. and of the free and conjugated forms 12.5 mg. per 100 ml. The level in cerebrospinal fluid was one-third to one-half the corresponding blood level.

All 10 patients with meningococcal meningitis treated by the drug alone and both with meningococcal meningitis treated by the drug and penicillin recovered without complications due to disease or treatment. One of the three patients with pneumococcal meningitis treated with the drug and penicillin died of pneumococcal endocarditis; the remaining two recovered as also did the two with *H. influenzae* meningitis treated with the drug and streptomycin. Gantrisin can be given orally, intramuscularly, or intravenously, alone or in combination with penicillin and streptomycin without any toxic effects. *Malcolm Woodbine.*

"Gantrisin" in the Treatment of Urinary Infections. CARROLL, G., ALLEN, H. N., and FLYNN, H. (1950). *J. Amer. med. Ass.*, **142**, 85.

Tests *in vitro* and *in vivo* have shown that "gantrisin," (3:4-dimethyl-5-sulphanilamido-iso-oxazole), also known as "NU-445," is the drug of choice in the treatment of urinary infections due to *Proteus vulgaris*. It is also effective in infections caused by *Bacterium coli, Alcaligenes,* and certain intermediate organisms, and moderately effective in infections caused by the *Aerobacter aerogenes* and paracolon bacillus. Infections due to *Pseudomonas* and *Streptococcus faecalis* are resistant to treatment. If one of the organisms in a mixed infection is a resistant organism the value of the drug is greatly reduced. When "gantrisin" is given orally in doses of 2 g. every six hours or by intramuscular or intravenous injection of a 40% solution, no local or systemic toxic reactions occur. The use of alkalis or forcing fluids at the same time is not necessary.

Thomas Moore.

Pertussis Treated with Chloramphenicol. PAYNE, E. H., LEVY, M., ZAMORA, G. M., VILARROEL, M. S., and CANELAS, E. Z. (1949). *J. Amer. med. Ass.*, **141**, 1298.

In the department of Cochabamba, Bolivia, whooping-cough is a serious disease carrying a mortality of 20%, which does not appear to be related to social status. It has been found that penicillin and streptomycin prevent complications, but do not shorten the disease. *Haemophilus pertussis* and *H. parapertussis* are both sensitive to chloramphenicol *in vitro*. In this paper is described the treatment with chloramphenicol of 50 patients under 5 years of age seriously ill with whooping-cough, the diagnosis being confirmed either by cough-plate or by

swab culture. The clinical history of seven of these cases is given. The drug was given by mouth, as a rectal suppository, or intravenously, and dosage was calculated according to body weight. The therapeutic results are reported as excellent, fever disappearing by the second day and the frequency of paroxysms decreasing considerably. The only complication seemed to be a slight nausea. Cough-plate cultures were negative after seven days' treatment. It would appear that the drug is specific, as the authors mention neither mortality nor complications in the treated cases.

Chloromycetin in the Treatment of Salmonella Enteritis. ROSS, S., BURKE, F. G., RICE, E. C., WASHINGTON, J. A., and STEVENS, S. (1950). *New Engl. J. Med.*, **242**, 173.

Nine cases of salmonella enteritis in infants were treated with "chloromycetin." The chloromycetin sensitivities of the various strains of salmonella isolated from these patients ranged from 2 to 5 μg. per ml.

In spite of the ready susceptibility of the organism to the drug, only two of the nine patients in this series became permanently free of the pathogen after treatment. In the other seven cases a transitory inhibitory effect on both the salmonella organism and the normal stool flora was observed; however, within five to 40 days after chloromycetin was discontinued the pathogen reappeared in the stools. No increase in chloromycetin resistance was observed in any case after bacteriologic relapse. —(Authors' summary.)

Chloramphenicol in Treatment of Infantile Gastro-enteritis. A Preliminary Report. ROGERS, K. B., KOEGLER, S. J., and GERRARD, J. (1949). *Brit. med. J.*, **2**, 1501.

A serologically specific type of *Bacterium coli* was found in the stools of 86 infants under the age of 1 year admitted to the Birmingham Children's Hospital during a 10-month period. In 25 of the 86 cases reported the appearance of this organism was followed by no untoward symptoms during one month's observation. In the remaining 61 cases a gastro-intestinal disturbance developed, varying from severe gastro-enteritis to mild diarrhoea, from which 13 of the patients died. All severe cases were treated with intravenous alimentation until the diarrhoea and dehydration were controlled, when oral feeding was gradually resumed. Initially, penicillin and sulphonamides were administered, but although they helped to control parenteral infections they had little or no effect on the gastro-enteritis. Twelve patients were treated with streptomycin with disappointing results.

Treatment with choramphenicol in 15 cases was much more satisfactory. All strains of *Bact. coli* B.G.T. tested were sensitive to this drug and also to aureomycin, but the former was chosen for therapeutic trial because of its relative stability. In most cases, the diarrhoea ceased and the organism disappeared from the faeces within two to eight days of starting treatment. In two cases, it reappeared one and seven days respectively after stopping treatment, but the organisms were still sensitive to chloramphenicol. Three children became carriers and in two of them chloramphenicol failed to eradicate the organism from the faeces. Apart from sore buttocks in four cases no other possible toxic reactions were observed.

Treatment of Typhoid Fever with Chloromycetin. Results in Four Cases and in a Chronic Carrier. COLLINS, H. S., and FINLAND, M. (1949). *New Engl. J. Med.*, **241**, 556.

From their limited experience the authors consider that, except for a lesser incidence of toxic effects, chloramphenicol is not strikingly more effective in this disease than aureomycin. It is suggested that earlier diagnosis, and possibly the use of maximal doses from the start, might give better results in some cases, but they are evidently disappointed with the drug.

Aureomycin Treatment of Herpes Zoster. FINLAND, M., FINNERTY, E. F., COLLINS, H. S., BAIRD, J. W., GOCKE, T. M., and KASS, E. H. (1949). *New Engl. J. Med.*, **241**, 1037.

This paper records the results of aureomycin treatment in 24 cases of herpes zoster. Administration was oral in 22 and the optimum dosage was found to be 1 g. four times a day, reduced to 0.5 g. for each dose after definite improvement or if there was nausea and vomiting. This was continued until the lesions showed signs of healing. The total dosage varied from 7.5 to 54 g. over 3 to 17 days, with an average of 19 g. in six and half days. Two patients who refused oral therapy were given 500 mg. in 500 or 1,000 ml. of 5% dextrose by slow intravenous drip, about one hour being taken for each dose.

Rapid healing usually began within about 24 hours and was complete in the majority of patients by the seventh or eighth day. In four

cases new lesions appeared during the first two days of therapy. In all cases pain was dramatically reduced in four or five days, but postherpetic pain was not prevented in six cases and lasted for from two weeks up to three months. The authors believe that aureomycin has a definite beneficial effect on the course of herpes zoster. *A. W. H. Foxell.*

The Association of Certain Types (α and β) of Bact. Coli with Infantile Gastro-enteritis. SMITH, J. (1949). *J. Hyg., Camb.*, **47**, 221.

A particular variety of coliform bacillus had been found to be associated with cases of infantile gastro-enteritis. This organism has been variously named the neapolitanum variation, D 433 (Taylor, Powell, and Wright), and type α (author's laboratory). During 1947 the α type was found to be associated with 94.7% of 207 cases of gastro-enteritis. In November, 1947, it was noted that the α type was less frequently isolated in specimens from clinical cases of gastro-enteritis. By the use of antisera for the "O," "H," and "K" (Kauffmann) antigens another fairly frequently occurring type was identified and was named by the author for convenience the β organism. Up to the end of 1947 the β type was found in 21 cases of infantile diarrhoea. The β variety has no distinctive features when grown on agar or blood-agar plates. (The original article should be consulted for full technical details.)

During 1948, 75 cases were classified definitely as cases of gastro-enteritis. The α type was recovered from 25 cases (six deaths) and the β type from 35 cases (11 deaths). In 14 cases (six deaths), clinically of gastro-enteritis, neither type was isolated. One patient died before bacteriological examination could be made. The α and β organisms are easily identified by specific slide-agglutination tests, but there are considerable technical difficulties in recovering the types from faecal cultures, particularly if the agglutinable types are scanty. Either organism can be readily isolated during the acute phase of the disease, becomes increasingly scanty in the sub-phase, and usually tends to disappear from the intestinal contents as convalescence is established. A certain number of convalescent carriers were observed.

The author considers that while the actual significance of the association of the α and β types with gastro-enteritis has not been determined, their isolation points to a diagnosis of infantile gastro-enteritis, and also provides a means of controlling nursing technique and determining whether or not intestinal cross-infection has occurred in cubicles or open wards.

The Relation of Staphylococcus Pyogenes to Infantile Diarrhoea and Vomiting. MARTYN, G. (1949). *Brit. med. J.*, **2**, 1146.

The following three groups of infants aged 2 weeks to 1 year were studied: (1) 119 infants in hospital suffering from diarrhoea and vomiting; (2) 78 infants in hospital, but without gastro-intestinal symptoms; and (3) 44 healthy infants attending a welfare centre.

Rectal swabs were taken in each case and were plated on to horse-blood agar plates and inoculated into 10% sodium chloride meat medium (see Maitland and Martyn, *J. Path. Bact.*, 1948, **60**, 553). All staphylococci grown were tested for coagulase production and positive strains referred to as *Staphylococcus pyogenes;* staphylococci were also tested for sensitivity to penicillin (40 units per ml.) and streptomycin (1,000 units per ml.). The frequency of isolation of *Staph. pyogenes* in each group was as follows: (1) 44.5%, 28.3% of these organisms being penicillin-resistant and 3.8% streptomycin-resistant. (2) 52.5%, 31.9% being penicillin-resistant and 4.9% streptomycin-resistant. (3) 34%, 60% being penicillin-resistant and none streptomycin-resistant. Twenty strains were not typable.

Distribution of bacteriophage types in the strains isolated was strikingly similar in patients (group 1) and controls (groups (1) and (2)); 19 in each (out of 53 and 56 strains respectively) being of type 47 (nomenclature of Central Public Health Laboratory). Of these 38, 26 were penicillin-resistant. In each series, five strains were of type 6/47 which has been shown by Allison (*Proc. R. Soc. Med.*, 1949, **42**, 216) to be associated with food poisoning.

In only four cases were staphylococci isolated direct on blood agar, suggesting that the number present was small; the selective medium, on the other hand, gave a high incidence of the organism from a sparse inoculum. In only four out of 13 fatal cases was *Staphylococcus pyogenes* isolated on selective medium. There was no blood or pus in the stools in any of the cases of group (1). The conclusion is drawn from the closely corresponding incidence of the organism in patients and controls and the similarity of phage-type distribution and sensitivity to penicillin and streptomycin that *Streptococcus pyogenes* is not the cause of infantile diarrhoea and vomiting.

A New Haemorrhagic Ulcerative Disease and its Aetiology. CASTELLANI, A. (1949). *J. trop. Med. Hyg.*, **52**, 204.

This paper records in detail observations on the clinical picture and bacteriology of two cases of a hitherto undescribed disease. The patients were seen late in the course of the disease. Bullae appeared, chiefly on the limbs and were filled with a haemorrhagic fluid. These ruptured leaving an ulcer which, on healing, produced a raised keloid scar. A low-grade fever was present and one patient had bouts of abdominal pain which were followed by the passage of haemorrhagic stools. A well marked hypochromic anaemia was present, the leucocytes being normal in number and distribution. *X*-ray and pathological examinations gave negative results. Treatment with sulphonamides and penicillin had little effect. The first patient was not followed up ; the second showed slow improvement over the course of a year.

The same organism was isolated both from the ulcers and from the bullae in each case. It grew well on the usual laboratory media and its cultural characteristics are described. Mice were readily killed by intraperitoneal injection of the freshly isolated organism. Injection into the skin of rabbits, guinea-pigs, and monkeys resulted in lesions which resembled those seen in man—especially in the case of monkeys. The organism is rod-shaped, 1.5 to 3.5 μ (or more) in length, non-sporogenous, actively motile, and easily stained. It was Gram-negative and not acid-fast. It showed some features akin to those of *Malleomyces pseudomallei*, and also to those of the genus *Pseudomonas*. The author proposes the name *Bacterium haemorrulcogenes*.

Q Fever in Great Britain. Clinical Account of Eight Cases. HARMAN, J. B. (1949). *Lancet*, **2**, 1028.

A clinical description is given of eight cases of Q fever occurring at the Royal Cancer Hospital, London. This is the first time that Q fever has been detected in the active state in Great Britain, although it is known that many cases arose in Italy and the Balkans during the late war, where they were called "Balkan grippe" by the Germans and "atypical pneumonia" by the British. The clinical manifestations, although constant, do not serve to distinguish Q fever from other short-term fevers ; it has usually drawn attention to itself by its epidemic character. Features of the clinical picture are high swinging temperature with rigors and drenching sweats, intense headache, and, quite commonly, delirium. Within a week a tickling unproductive cough develops and tissue-paper crepitations and high-pitched bronchial breathing are to be heard usually at the base. Gastro-intestinal symptoms are rare. There is no rash or coryza. The radiological signs, which may persist for weeks, suggest lobular partial consolidation usually at the base, though any lobe may be affected ; collapse and pleural effusions may be seen. Complications in this disease are hardly ever seen and the death rate seems to be in the neighbourhood of 1 in 500. Case-to-case spread is remarkably absent.

Diagnosis was confirmed in all the eight cases except one (probably the original one) by the complement fixation test for *Rickettsia burnetii*. Antibody titres as high as 1 in 640 were recorded. In one case *R. burnetii* was isolated from the blood. In treatment the sulphonamides, penicillin, and probably streptomycin are useless. Aureomycin, which has a powerful suppressive action on experimental infections of eggs and guinea-pigs with the rickettsia of typhus, scrub typhus, Rocky Mountain spotted fever, and Q fever, has been used clinically in cases of Q fever with promising results, though one notable failure was seen and in others cure was delayed. Chloramphenicol is very effective in other rickettsia diseases, but there are no published records of its use in Q fever.

J. V. Armstrong.

Antihistamines in the Treatment of the Common Cold. A Preliminary Report. MIDDLETON, J. W., and RIDER, J. A. (1949). *Dis. Chest*, **16**, 879.

Random treatment of the common cold with antihistaminic drugs indicated that some improvement occurred, and a controlled experiment was therefore made, 63 cases being treated with antihistaminic drugs and 29 with placebos. The drugs used were pyranisamine and phenindamine, and these were given four times daily in 50 mg. and 25 mg. doses respectively. No patient knew what drug was being administered. Drugs were assigned alternately in the order in which patients appeared.

There was no significant difference in results from pyranisamine and phenindamine, though the former gave rise to a higher percentage of side-effects. None of these was severe. Patients with a history of allergy were tabulated separately. Colds were considered to be aborted

only if symptoms were entirely relieved within 12 hours after the start of therapy and remained absent after discontinuation of the latter. Of the total treated 76% were benefited, with partial or complete relief of symptoms, as compared with 45% of controls. In only seven cases was the cold aborted. Of those with a history of allergy 88% were benefited as compared with 72% of the non-allergic (though the former group was small and may have included cases of allergic rhinitis and not genuine colds).

The authors do not claim any altered response to viral or bacterial infection, but rather a modification of symptoms.

Treatment of Surgical Tuberculosis with the Thiosemicarbazone "TB I 698." HASCHE-KLÜNDER, R., and LEIMBACH, G. (1949). *Dtsch. med. Wschr.*, **74**, 1135.

Thiosemicarbazone as a chemotherapeutic agent has been used mainly in the treatment of pulmonary tuberculosis, and there are few reports of its use in surgical tuberculosis. The authors report their experience in 70 cases of non-pulmonary tuberculosis treated with thiosemicarbazone. Although no response was obtained in some cases, there was often marked improvement in the general condition of the patient, with a quicker and better localization of the active process, so that surgery could be employed earlier and with more certain healing. The drug was given by mouth and also locally by instillation into fistulae or abscess cavities.

It was most effective in bladder, bone, and joint tuberculosis, and in fistula-in-ano, but no effect was observed on cases with lesions of the kidney, prostate, and epididymus.

The authors point out that very careful experiment and observation are required for the evaluation of these drugs and the assessment of the indications and contraindications for their use before they can be accepted and related to other substances, such as streptomycin, in the treatment of tuberculosis.

HAEMATOLOGY

Diagnosis and Mechanism of Hemolysis in Chronic Hemolytic Anemia with Nocturnal Hemoglobinuria. DACIE, J. V. (1949). *Blood*, **4**, 1183.

The primary abnormality in nocturnal haemoglobinuria resides in the patient's erythrocytes. It is of such a nature as to cause them to be haemolysed by thermolabile fraction(s) of normal serum, but only if the *p*H of the serum is adjusted to the physiological level or below by the addition of acid to compensate for the alkalinity which develops when serum is exposed to air and loses carbon dioxide. This is the basis of the "acid-serum test." The patients' corpuscles are *not* unusually sensitive to acid *per se*.

An increase in haemolysis as the result of acidification of serum is, however, not specific for the reaction in nocturnal haemoglobinuria; other types of haemolysins are affected by change in *p*H and may be a cause of confusion. The necessary controls are outlined.

The nature of the corpuscular abnormality and the haemolytic factor(s) present in normal serum is briefly considered.

Acquired Hemolytic Anemia. I. The Relation of Erythrocyte Antibody Production to Activity of the Disease. II. The Significance of Thrombocytopenia and Leukopenia. EVANS, R. S., and DUANE, R. T. (1949). *Blood*, **4**, 1196.

The authors have studied patients with acquired haemolytic anaemia whose corpuscles give a positive Coombs test. They have attempted to equate the intensity of haemolysis as estimated clinically with the degree of sensitization of the corpuscles, as measured by ascertaining the highest dilution of antiglobulin serum which would give a positive reaction. In general low titres of antiglobulin serum were associated with clinical quiescence, both before and after splenectomy. In one case, however, this was not so, for the corpuscles remained strongly sensitized without there being clinical evidence of a return of haemolysis.

Sickle Cell Anaemia, a Molecular Disease. PAULING, L., ITANO, H. A., SINGER, S. J., and WELLS, I. C. (1949). *Science*, **110**, 543.

The authors have studied the haemoglobin molecule by physical means and have found that there is a difference in electrophoretic behaviour between haemoglobin of normal subjects and that derived from patients suffering from sickle cell disease. Corpuscles from subjects with the sickle cell trait contain both types of haemoglobin. The molecular abnormality is believed to affect the globin part of haemoglobin and to be of such a nature as to allow alignment of complementary parts of the molecules when oxyhaemoglobin is changed to reduced haemoglobin.

Pteroyl-polyglutamic Acids in the Treatment of Pernicious Anaemia. WILKINSON, J. F., and ISRAËLS, M. C. G. (1949). *Lancet*, **2**, 689.

Thirteen patients were treated with synthetic pteroyl-γ-triglutamic acid (teropterin) and/or pteroyl-α-diglutamic acid (diopterin) by the intravenous, intramuscular, and oral routes. In 8 patients treatment with teropterin alone produced satisfactory haematological responses, while in 3 patients treated with minimal doses of diopterin the response was less.

These results are held to indicate that patients with pernicious anaemia can hydrolyse and utilize synthetic folic acid.

Erythropoietic Effect of Cobalt in Patients With or Without Anemia. BERK, L., BURCHENAL, J. H., and CASTLE, W. B. (1949). *New Engl. J. Med.*, **240**, 754.

The results of cobalt administration to patients with a variety of anaemias proved disappointing. In none of 5 patients with refractory anaemia and hypocellular marrow, nor in patients with anaemia associated with nephritis or liver disease was there a significant response. A definite rise in the reticulocyte level and in the erythrocyte count was observed, however, in 2 of 5 patients with chronic infections, in 1 or 2 patients with inoperable carcinoma of the stomach and in one patient with Cooley's anaemia. A slight reticulocyte response was produced in 17 patients with a normal blood picture who were given 300 mg. cobaltous chloride daily.

Idiopathic Thrombocytopenic Purpura. ROBSON, H. N. (1949). *Quart. J. Med.*, **18**, 279.

Nineteen cases of thrombocytopenic purpura submitted to splenectomy were studied, as well as 14 patients undergoing splenectomy for other conditions. Seven patients with no blood abnormality who were undergoing abdominal operations served as controls.

In thrombocytopenic purpura the bleeding time was reduced as soon as the skin incision was made; it reached normal on an average 48 minutes after ligation of the splenic pedicle. Capillary resistance increased after ligation of the splenic pedicle and became normal about 4½ hours later. The platelet count also increased, reaching normal levels in ½–4 hours and maximum levels up to 22 days afterwards. Observations on the control group suggest that the changes in bleeding time and capillary resistance and platelet counts are non-specific and due to the operation itself. Later changes are effected by the removal of the spleen. The megakaryocytes were also studied; lack of platelet formation was the most constant finding.

The Cause of Thrombocytopenia in Sedormid Purpura. ACKROYD, J. F. (1949). *Clin. Sci.*, **8**, 269.

One or two tablets of sedormid taken by mouth by patients who have recovered from purpura caused by this drug may precipitate another attack of thrombocytopenia. In such patients the drug causes agglutination and lysis of platelets *in vitro* and capillary damage if applied to the skin. The minimal concentration of sedormid required to cause lysis of platelets *in vitro* corresponds closely with that calculated to be present in the plasma after therapeutic doses of the drug.

The Control of Polycythemia by Marrow Inhibition. A Ten Year Study of 172 Patients. LAWRENCE, J. H. (1949). *J. Amer. med. Ass.*, **141**, 13.

One hundred and twenty-one patients with polycythaemia vera have been treated with P_{32} during the past 12 years. They received as a rule 2 injections of 3 to 6 millicuries of radioactive phosphorus. This course was usually repeated after an average of 3 years, but many patients needed no further treatment for 4–8 years. Occasionally this regime has been supplemented by venesections. Twenty-one of the patients died, 5 of leukaemia, a recognized complication of polycythaemia (however treated). The incidence of thrombosis during treatment was small, and it is concluded that P_{32} therapy is a satisfactory way of treating polycythaemia vera.

Homologous Serum Jaundice. LEHANE, D., KWANTES, C. M. S., UPWARD, M. G., and THOMSON, D. R. (1949). *Brit. med. J.*, **2**, 572.

The occurrence of jaundice following transfusions was ascertained in a series of 4,430 patients. The incidence after blood transfusion (0.8%) was not significantly different from that following transfusion of plasma obtained from small (10 donor) pools (1.3%). The incidence after transfusion of plasma prepared from large pools was much greater (11.9%).

J. clin. Path. (1950), 3, 301.

THE LABORATORY DIAGNOSIS OF HAEMOPHILIA

BY

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Haemophilia is the only recognized haemorrhagic diathesis which is inherited as a sex linked recessive character. It is a disease of males which is transmitted through apparently unaffected females. The classical case can be recognized with certainty since the laboratory manifestations are specific. The association of a prolonged coagulation time with a normal bleeding time and platelet count, if combined with a family history of a bleeding tendency, can hardly ever lead to any other diagnosis.

During an investigation of the nature of the defect in haemophilia it was possible to examine a large number of cases of haemorrhagic disease. It soon became apparent that the laboratory diagnosis of the haemophilic state was not as simple as had at first been assumed. Of the cases which had a familial and clinical history resembling haemophilia, many had typical laboratory findings, yet in other apparently similar cases it was difficult to obtain laboratory confirmation of the disease. Accordingly, it seemed worth while to review the pathological findings in haemophilia in an attempt to assess their significance and value in diagnosis. It is these observations which form the basis of this paper.

Material

The material for this series was derived from the records of a number of the London teaching hospitals and those of the Radcliffe Infirmary. The case records of Dr. R. G. Macfarlane and Professor L. J. Witts provided additional cases, and also a certain number of patients were referred to this hospital from other centres, either because our interest in the subject was known or alternatively because of difficulty in diagnosis. All cases which fulfilled our diagnostic criteria for haemophilia were included in this series. In all, 54 haemophilics were actually seen and examined, and records of another 18 were available for analysis.

Familial Incidence

The familial incidence of the present series is shown in Table I.

The typical family history is a valuable clue to diagnosis of haemophilia, but the occurrence of apparently sporadic cases has often been reported, and haemophilia can be diagnosed in the absence of a family history. Some of these apparently

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TABLE I

INCIDENCE OF FAMILIAL HISTORY OF HAEMOPHILIA IN PRESENT SERIES

	Patients with Family History		Patients with No Family History
	Patients	Families	
Patients seen and examined	37	23	17
Patients from Radcliffe Infirmary records ..	7	6	11
Totals	44	29	28

sporadic cases may arise from silent transmission of the bleeding tendency through a long line of carrier females; others may arise by mutation.

It will be seen that in 28 out of 72 patients of the present series (i.e., 39%) no family history of bleeding could be elicited. Twenty-nine out of a total of 57 families had more than one affected member. Birch (1937) reported 51 out of a total of 73 families with a familial history of bleeding disease. The greater incidence of isolated cases in the present series may be due to selection, since only seven patients were normally resident in Oxfordshire. Furthermore many cases of haemorrhagic disorder without a family history were referred to us for confirmation of the haemophilic state because the absence of such a history was regarded as a strong point against the diagnosis.

Laboratory Manifestations

Bleeding Time, Platelet Count, and Tourniquet Test.—No extensive observations were made on these three tests, since almost invariably normal results were obtained. On one occasion, in an undoubted haemophilic, a positive tourniquet test was encountered. On one occasion, too, a long bleeding time was found. This served to emphasize the fact that if, in performing the test for bleeding time in haemophilia, vessels other than small capillaries are damaged, bleeding may not cease in the normal range of time of this test.

Coagulation Time.—The lengthening of the coagulation time has been rightly regarded as the classical laboratory abnormality in haemophilia. This is also reflected in the lengthening of the clotting time of recalcified blood, which seems to be peculiarly sensitive to the number of platelets present, as Gram first showed in 1920. Quick (1941) took advantage of this phenomenon in elaborating the so-called Quick's test for haemophilia. Finally, if all the platelets are removed from the plasma the blood becomes completely incoagulable (Conley, Hartmann, and Morse, 1949).

Prothrombin Consumption.—Brinkhous (1939) first showed that, when haemophilic blood clotted, the consumption of prothrombin was less than normal, and this has since been confirmed (Quick, 1947; Soulier, 1948; Alexander and de Vries, 1949; Merskey, 1950a). Prothrombin consumption can be measured on the serum from whole clotted blood, obtained by venepuncture or finger-prick, or on that obtained by recalcifying citrated plasma.

Antihaemophilic Globulin.—If a small proportion of normal blood which contains the substance usually known as antihaemophilic globulin be added to haemophilic blood, all the defects so far mentioned are largely corrected, and this characteristic reaction is used as a test for haemophilia. Conversely, the blood from a patient suspected of having haemophilia can be added to known haemophilic blood and its corrective ability compared with that of normal blood.

The essential feature of this last test is that the clotting efficiency of a mixture of two haemophilic blood samples is unlikely to be greater than either separately, and in no case can this mixture be as good as either of these haemophilic blood samples mixed with an equivalent proportion of normal blood.

Laboratory Techniques

Coagulation Time.—Coagulation times were measured on venous blood (Lee and White, 1913) and finger-prick blood (Dale and Laidlaw, 1911–12), the latter having been shown to be the better of two methods which used capillary blood (Biggs and Macmillan, 1948).

Venous Blood.—This was collected in a paraffined syringe and 1 ml. placed in each of four dry test-tubes ($2\frac{1}{2}$ in. $\times \frac{3}{8}$ in.) which were kept at 37° C. The tubes were gently tilted every half minute and the time from the first appearance of blood in the syringe to coagulation of the blood was recorded. If difficulty were encountered with venepuncture or if bubbles appeared in the syringe the result was rejected as unreliable.

Capillary Blood.—A small tube of wide capillary bore containing a freely mobile lead shot (average weight 0.006 g.) was prepared. A sharp needle was used to prick the finger and the first drop of blood which emerged was used to fill this capillary tube. The tube, held by rubber-covered forceps in a water bath at 37° C., was tilted every 10 seconds and the time between filling the capillary and that at which the lead shot ceased to move was recorded.

Recalcified Plasma.—Blood was collected into 3.8% sodium citrate, using one part of citrate to nine parts of blood. Plasma was obtained by centrifuging the blood at 1,500 revolutions per minute for five minutes. Recalcification was effected at 37° C. by the use of equal volumes of plasma, normal (0.85%) sodium chloride solution, and M/40 calcium chloride solution, and the time of the tubes to clot was recorded These tests were performed in duplicate and the results averaged.

Quick's Test (1941) for Haemophilia.—Oxalated blood, 5 ml., was divided into two equal portions. One of these was centrifuged at 750 r.p.m. for five minutes. The second was centrifuged in an angle centrifuge at 3,000 r.p.m. for a similar time. Samples, 0.1 ml., of each of these plasmas were recalcified at 37° C. with 0.2 ml. of M/80 $CaCl_2$ (0.14 g. $CaCl_2$, 0.42 g. sodium chloride in 100 ml. distilled water) and the time taken for the mixture to clot was recorded in each case. The test was done in quadruplicate and the results averaged. All procedures were completed within 30 minutes of taking the blood.

Prothrombin Consumption Index (P.C.I.).—This was measured by a two-stage technique (Merskey, 1950a). The test is based on the measurement of the amount of prothrombin present in citrated plasma compared with the amount present in the serum of blood in glass tubes one hour after coagulation had occurred.

Serum from Whole Clotted Venous Blood.—The serum from the four Lee and White coagulation tubes was pooled and a sample tested.

Serum from Whole Clotted Capillary Blood.—Blood was obtained by finger-prick and the finger was squeezed in order to obtain a mixture of blood and tissue fluid.

Serum from Recalcified Citrate Plasma.—Plasma was obtained from citrated blood by centrifuging the blood at 1,500 r.p.m. for five minutes. It was recalcified with an equal volume of M/40 $CaCl_2$.

In all cases the tubes were incubated for one hour at 37° C. and the serum obtained by centrifuging in the case of whole blood, or by expression of the clot in the case of recalcified citrate plasma. In the latter case an allowance was made for the dilution with $CaCl_2$.

In the measurement of prothrombin 0.2 ml. each of brain thromboplastin, M/40 $CaCl_2$, normal saline, and serum (the serum being added last) were incubated in a water bath at 37° C., and 0.1 ml. samples removed at 20 seconds, 60 seconds, and 120 seconds thereafter and added to a second row of tubes each containing 0.4 ml. fibrinogen. The time taken for the latter tubes to clot was recorded. The plasma was treated in a similar manner and the prothrombin content of the serum was compared with that of the original plasma and expressed as a prothrombin consumption index as follows:

$$\text{P.C.I.} = \frac{\text{most rapid plasma time}}{\text{most rapid serum time}} \times 100$$

Correction of Haemophilic by Normal Blood.—The known haemophilic, the normal control, and the patient to be tested, all being of compatible ABO blood groups were venepunctured simultaneously using similar sized needles and syringes for obtaining the two latter specimens. The blood of the haemophilic and the normal control, or the haemophilic and the patient to be tested, was mixed in the proportions shown in Table II (there being approximately 40 drops per ml.). Each tube was inverted once and 1 ml. of each mixture was placed in a glass tube $2\frac{1}{2}$ in. $\times \frac{1}{8}$ in. and the time taken for the contents of each tube to coagulate at 37° C. determined. One hour later the prothrombin remaining in each tube was measured.

TABLE II

DILUTION SCHEME FOR TITRATION OF ANTIHAEMOPHILIC GLOBULIN CONTENT OF BLOOD

Volume of haemophilic blood (ml.)	1.0	—	1.0	1.0	1.0	1.0	1.0	2.0	4.0	8.0
Volume of patient's (or control) blood (drops)	—	40	16	8	4	2	1	1	1	1
Concentration of patient's (or control) blood in haemophilic blood (%)	0	—	29	17	9	5	2.5	1.25	0.6	0.3

Recalcified Citrate Plasma.—Blood samples from a known haemophilic, the patient to be tested, and a normal control were obtained within a short time of each other. They were citrated as before and centrifuged at 2,000 r.p.m. for five minutes to obtain plasma. Dilutions of plasma of the patient to be tested and the normal control were made separately in normal saline, suitable dilutions being 0.75%, 1.5%, 3%, 6%, 15%, and 30%. Of each of these dilutions 0.2 ml. was added separately to 0.2 ml. known haemophilic plasma, and this was recalcified with 0.2 ml. M/40 $CaCl_2$ and the calcium time at 37° C. determined. The final dilutions would then be one-third of the above mentioned figures. Dilutions of haemophilic plasma with 0.2 ml. saline were also recalcified at the beginning and end of the experiment. All observations were made in duplicate. One hour later the prothrombin content of the serum in each tube was measured.

Results

Coagulation Time in Normals.—The results for venous blood in 25 normal males and 25 normal females are shown in Fig. 1, where each tube has been plotted separately. There is a slight difference between males and females which is statistically significant, but for an individual coagulation time this difference can be ignored.

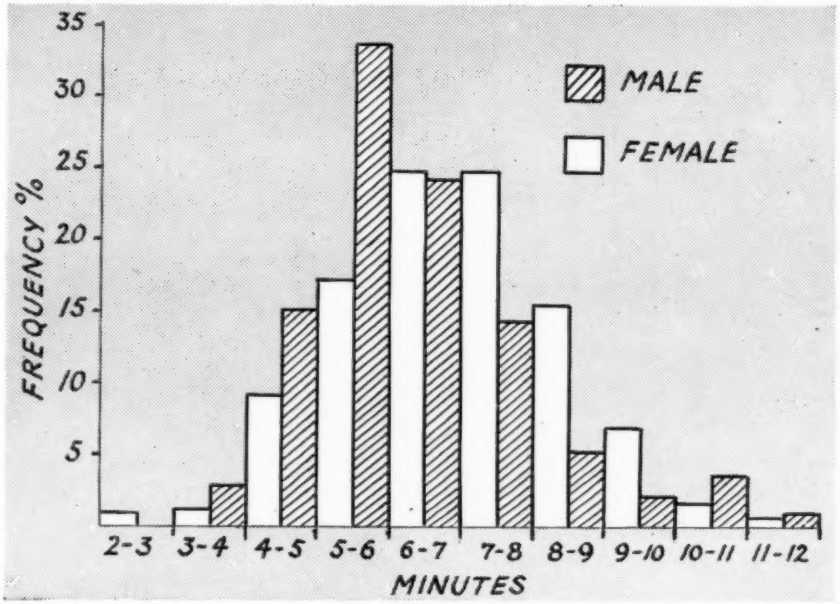

FIG. 1.—Histogram showing results of Lee and White coagulation times in 25 normal males and 25 normal females. The four tubes of each test have been plotted separately.

The range was found to be from two to 12 minutes for individual tubes and from three and three-quarters to 10 minutes for the average for any particular individual. An average for the four tubes in excess of 11 minutes is regarded as abnormal.

The results for capillary blood in 25 normal males and 25 normal females are shown in Fig. 2, and the mean figures and standard deviation in Table III.

TABLE III

COAGULATION TIME (DALE AND LAIDLAW METHOD) IN NORMAL CONTROLS

	Mean (Seconds)	Standard Deviation (Seconds)
Males	124.1	15.8
Females	131.8	14.8
Whole group	127.9	15.7

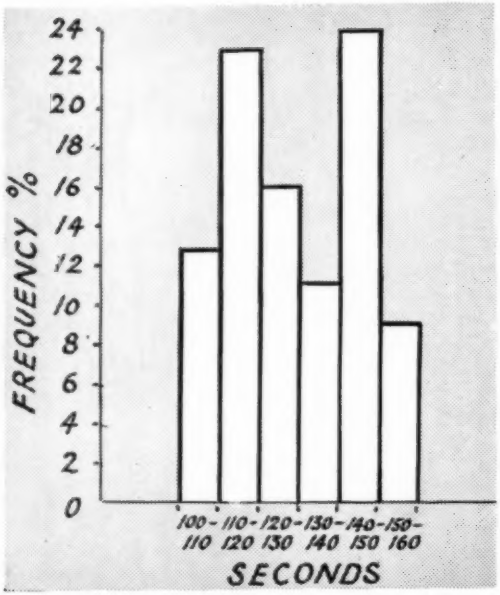

FIG. 2.—Histogram of results of Dale and Laidlaw coagulation times in 50 normal individuals.

Dale and Laidlaw (1911–12), using a slightly larger average shot, found an average clotting time of 101.5 seconds with a mean error of 4.3 seconds. Abrahams and Glynn (1949) tested 16 normals and found a range from 120 seconds to 210 seconds with an average of 165 seconds, but the temperature at which these observations were made was not specified. It is obvious, therefore, that minor alterations in technical detail, and especially in the bore of the capillary tube and the size of the lead shot, may affect the results, and individual laboratories should evaluate their own range of normal. In this laboratory figures in excess of 180 seconds are regarded as being outside the normal range.

Coagulation Time in Haemophilia.—In many cases of haemophilia the coagulation time by any reliable method is greatly prolonged. In 10 patients of the present series the venous blood coagulation time fell within the normal range; in 11, it was just over the upper level of normality; and it was well beyond the normal range in the remaining 30 patients (Fig. 3).

Reliability of Dale and Laidlaw Method.—Quick, Honorato, and Stefanini (1948) make the statement:

> "It must be emphasized that the coagulation time of capillary blood, irrespective of the method used, is worthless and unreliable for clinical purposes."

Even admitting the superior reliability of the coagulation time of venous blood, this criticism of capillary blood methods seems unduly harsh. To investigate the point further the Dale and Laidlaw coagulation time of each case was compared with the Lee and White time to see how often a prolonged coagulation time would be observed by either method.

Consecutive Tests on Same Patients.—Two haemophilics with prolonged Lee and White coagulation time were tested approximately every two days over a two to three week period. The results are shown in Fig. 4. It will be seen that the Lee and White time was prolonged on all occasions, whereas on four occasions the Dale and

Laidlaw method gave results which were within normal limits. Immediate repetition of three of these Dale and Laidlaw estimations gave figures which were definitely abnormal. The fourth was unfortunately not repeated.

Fifty-two Parallel Observations on 46 Haemophilics.—Six patients were re-examined on a second visit to the hospital. It will be noted that the Dale and Laidlaw method failed to disclose a greatly prolonged coagulation time in four instances when the Lee and White time was over 15 minutes. Three of these four were immediately repeated, and the readings were 420, 210, and 165 seconds. Thus the Dale and Laidlaw method might be misleading in two of these 25 cases. When the Lee and White coagulation time was 15 minutes or less the capillary blood method seemed to be at least as reliable as the method using venous blood. Occasionally, definitely abnormal readings were obtained with the Dale and Laidlaw method, when the Lee and White coagulation time was normal. In these cases the capillary blood method appeared to give more information than the use of venous blood (Table IV and Fig. 5).

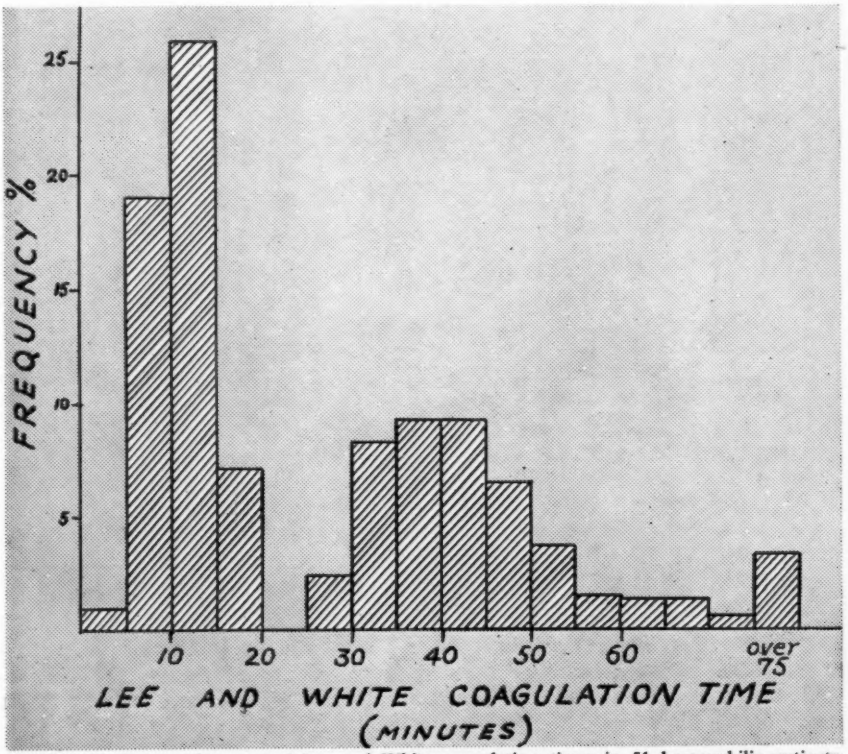

FIG. 3.—Histogram of results of Lee and White coagulation times in 51 haemophilic patients. The four tubes of each test have been plotted separately.

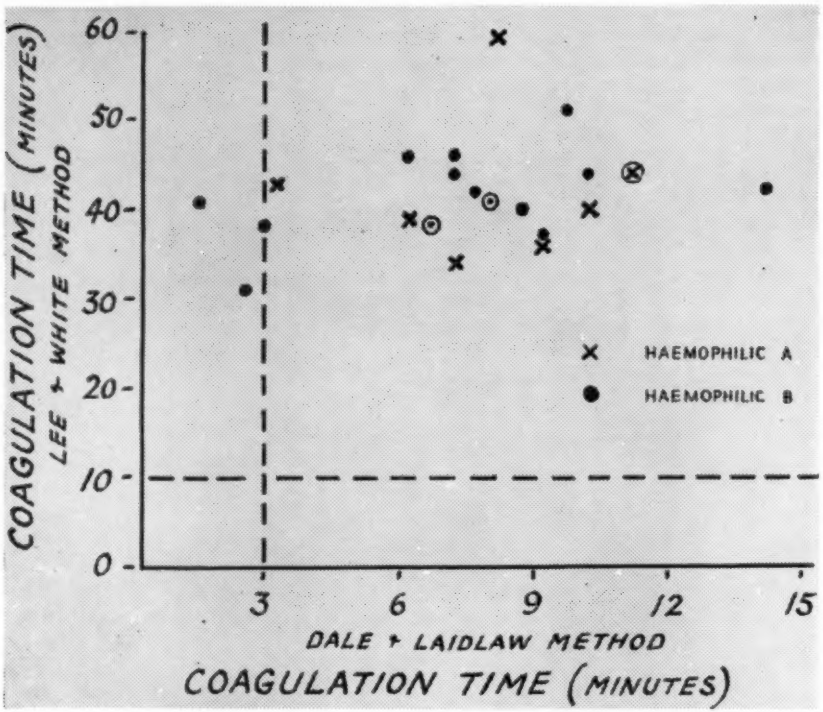

FIG. 4.—Comparison of two methods of measuring coagulation time. Two haemophilics with prolonged coagulation time tested every second day. Note that four readings by the Dale and Laidlaw method fall in or near the normal range, but repetition of three of them (circles) all fall well outside the normal range. The broken lines indicate the normal range of each test.

Recalcified Plasma.—Observations made on the clotting time of recalcified citrate plasma showed that, in general, this tended to be parallel to the Lee and White time. It was greatly increased when the Lee and White time was prolonged and even moderately lengthened in some of the cases where the Lee and White time was near normal. The figures obtained varied considerably, and, since the results were affected by the age of the plasma and the number of platelets present, it did not appear to be as reliable a method of evaluating a case of haemophilia as the use of whole blood without anticoagulant.

Quick's Test for Haemophilia.—The results of this test in 18 cases of haemophilia and 20 normal controls are shown in Fig. 6. The case records of the Radcliffe Infirmary were searched and another 10 examples of Quick's test in haemophilia discovered. These are also shown in the same diagram. In this diagram the percentage increase of the calcium time caused by high speed centrifugation has been plotted against the original (slow speed) calcium time. Some

TABLE IV
COMPARISON OF TWO METHODS OF MEASURING COAGULATION TIME

Lee and White Coagulation Time	Dale and Laidlaw Coagulation Time		
	Over 240 Seconds	180–240 Seconds	Under 180 Seconds
Over 15 minutes	21	3	1
10–15 minutes	8	6	4
Under 10 minutes	1	3	5

haemophilic blood samples were undoubtedly more affected by centrifuging than the normal controls, increases of 160%, 180%, and 191% being recorded in the three extreme cases. But in other cases very little increase was found. In the

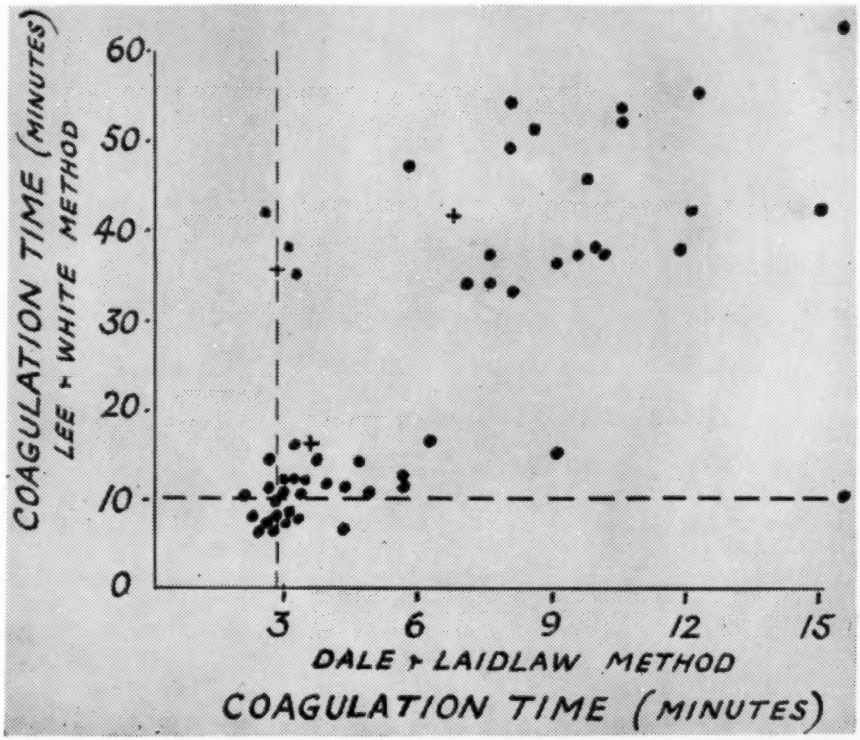

FIG. 5.—Comparison of two methods of measuring coagulation time (52 parallel determinations on 46 haemophilics). Note the close correlation recorded by the two methods, the Dale and Laidlaw method failing to indicate a prolonged coagulation time in only four instances. Three of these readings were immediately repeated (crosses). The broken lines indicate the normal range of each test.

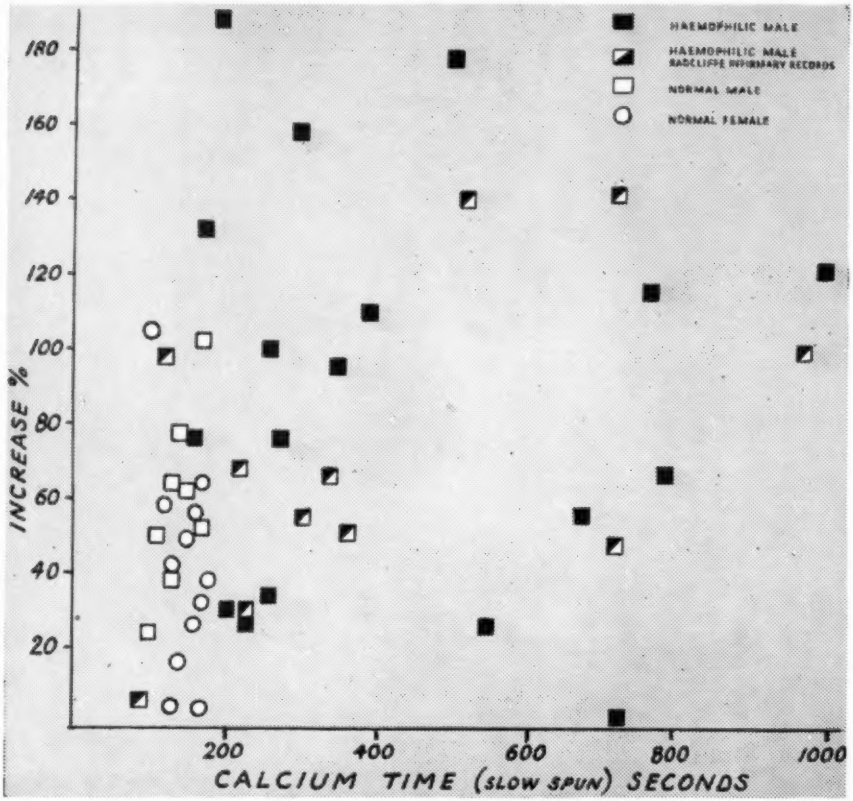

FIG. 6.—Results of Quick's test for haemophilia.

normals the increases were by no means negligible, and in two cases a normal was increased by over 100%, while in five cases the percentage increases exceeded 60%.

The percentage increase in the calcium time of nine cases of haemophilia was beyond the normal range. In 19 cases the increase was no greater than the normal range. The test was no more accurate in cases with a long as compared with those with a short calcium time. It was therefore only of slight value in the diagnosis of haemophilia.

Prothrombin Consumption Index (P.C.I.)—Under the conditions specified the P.C.I., using serum from whole clotted venous blood in normal controls, is usually less than 20% and always under 40% (Merskey, 1950a), while figures for serum from clotted capillary blood or recalcified plasma are usually less than this and often under 5%.

TABLE V

COAGULATION TIME AND PROTHROMBIN CONSUMPTION INDEX IN CASES OF THIS SERIES

No.	Age (Years)	Coagulation Time		Prothrombin Consumption Index (%)			Other
		Lee and White Method (Mins.)	Dale and Laidlaw Method (Secs.)	Whole Clotted Blood		Recalcified Citrate Plasma	
				Venous	Capillary		
1	16	150	1,140	108	100	135	
2	11	84	—	126	—	—	
3	13	55	735	200	110	200	
4	60	54	480	145	133	177	
5	50	53	630	150	—	—	
6	4	52	630	110	—	100	
7	59	51	510	133	—	125	
8	50	49	480	122	—	—	
9	50	48	—	71	—	—	
10	10	47	340	166	—	—	
11	4	42	580	166	187	150	
12	5	42	1,040+	150	188	157	
13	40	37½	150	158	85	137	Repeat Dale and Laidlaw reading 420
14	2	37½	708	212	190	190	
15	7	37½	605	183	122	147	
16	12	37	600	107	100	94	
17	44	37	450	210	100	100	
18	9	37	570	188	75	—	
19	42	36	540	136	125	108	
20	10	35	190	160	80	—	Repeat Dale and Laidlaw reading 165
21	7½	34	450	170	77	121	
22	39	34	420	188	155	—	
23	40	33	—	125	—	—	
24	4	33	480	180	—	—	
25	18	17½	370	100	12	79	
26	4	16	—	112	—	—	
27	10	16½	—	60	—	—	
28	13	16	195	100	<8	34	Repeat Dale and Laidlaw reading 210
29	7	15	540	190	100	—	
30	36	15	270	100	—	58	
31	36	14½	155	94	<5	115	
32	20	14½	300	146	14	25	
33	37	14	220	140	<5	156	
34	57	12½	332	44	<5	<5	
35	9	11½	330	48	6	80	
36	24	11½	260	83	<5	42	
37	29	11½	230	74	<5	31	
38	30	11½	190	64	<5	<5	
39	40	11½	185	64	<5	<5	
40	19	11¼	215	58	6	6	
41	37	11	165	35	<5	<5	
42	67	10¼	293	100	<5	<5	
43	24	10	205	60	12	25	
44	9	9¾	190	36	<5	45	
45	19	9½	200	47	<5	75	
46	19	8½	220	27	5	20	
47	38	8½	165	115	<5	<5	
48	17	8	152	41	12	12	
49	35	7½	155	65	10	71	
50	17	7	153	82	<5	7	
51	3½	6½	255	113	16	100	
52	2½	—	720+	—	At least 100		Venous blood unobtainable
53	20/12	—	680	—	At least 100		Serum prothrombin compared with normal plasma
54	9	—	460	—	At least 100		

FAMILIES: 3, 54; 4, 9, 27; 13, 17; 34, 38, 39, 41, 42; 35, 36, 37, 51; 40, 43, 45, 46.

Haemophilia.—The P.C.I. was found to vary in a manner roughly proportional to the coagulation time, and the readings for the whole series of cases are recorded in Table V and Fig. 7, where the cases have been arranged in relation to their Lee and White coagulation time.

It will be seen from Table V and Fig. 7 that the longer the coagulation time the more likely are all three methods of measuring the P.C.I. to be abnormal. As the coagulation time shortens, the capillary blood is the first to give normal readings.

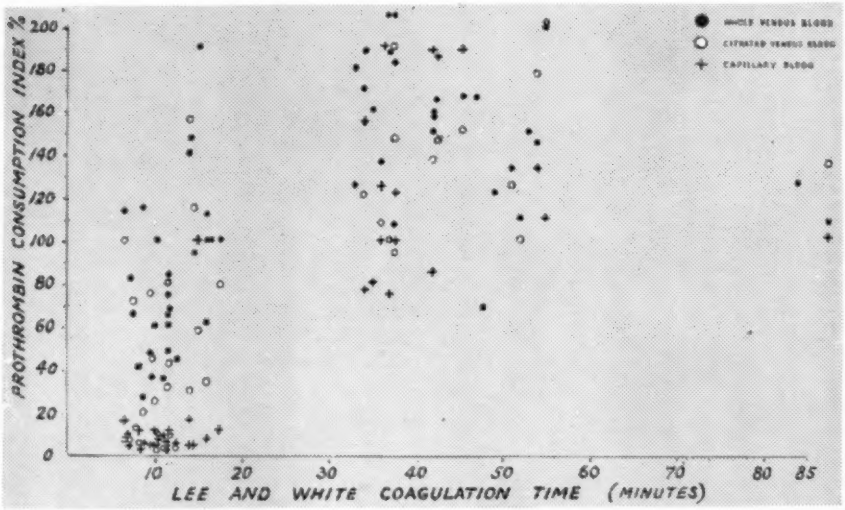

FIG. 7.—The prothrombin consumption index related to the Lee and White coagulation time in 51 haemophilic patients. Note that the P.C.I. falls as the coagulation time shortens. The use of serum from whole clotted venous blood is especially valuable when the coagulation time is in or near the normal range.

As it shortens further the recalcified citrate plasma readings become normal whereas the serum from whole clotted venous blood remains abnormal in the vast majority of instances including even cases with normal coagulation time. The degree of abnormality in the latter is less than in those with a long clotting time and approaches very close to normal in quite a number. In three instances it falls into the normal range.

Correction of Haemophilic Blood by Normal Blood.—The effect of the addition of increasing proportions of blood of one normal female and one normal male to that of a haemophilic with prolonged coagulation time is shown in Table VI. It will be seen that in both cases the coagulation time shortens progressively and the prothrombin remaining in the serum rapidly diminishes. There seems to be no significant difference between the ability of the blood of the normal male and the normal female to correct haemophilic blood.

TABLE VI

EFFECT ON COAGULATION TIME AND PROTHROMBIN CONSUMPTION INDEX OF HAEMOPHILIC BLOOD BY ADDITION OF NORMAL BLOOD

Percentage Blood Added	Lee and White Coagulation Time (Mins.)		Prothrombin Consumption Index (%)	
	Normal Male	Normal Female	Normal Male	Normal Female
Nil	34		188	
0.6	16½	13½	81	100
1.25	13½	11¾	81	71
2.5	11	11½	41	85
5.0	11¾	9¾	48	38
9.0	9	7¾	40	46
17.0	7½	7	38	31
35.0	7	6½	—	22
Control	6½	7½	<5	<5

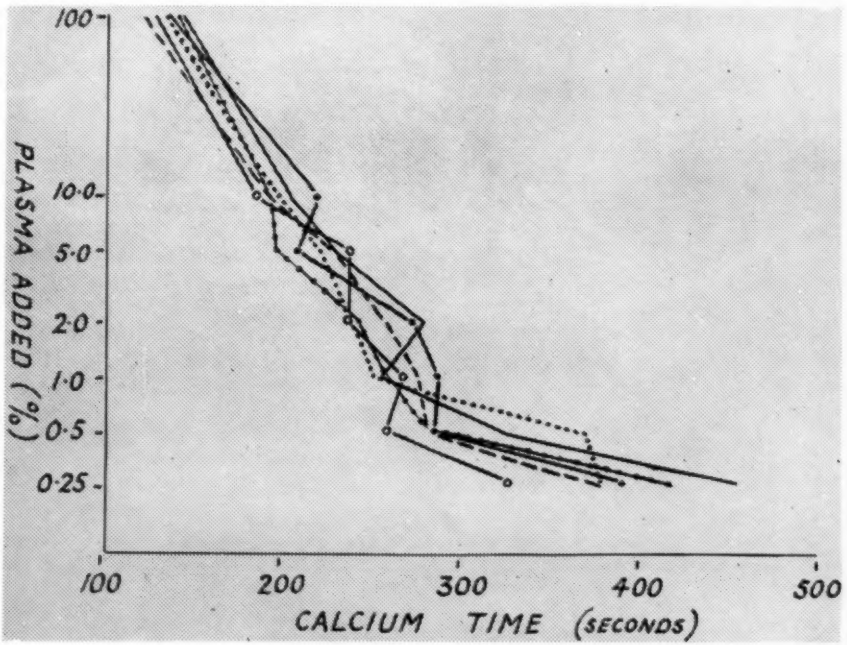

FIG. 8.—Shortening of the calcium time of known haemophilic plasma produced by plasma from six normal controls. The percentage of plasma added is plotted on a logarithmic scale. The calcium time of the haemophilic plasma alone was 1,230 seconds.

Recalcified Citrate Plasma.—The ability of six normal controls (four males and two females) to correct the calcium time of haemophilic blood is shown in Fig. 8. In addition the effect of each of these on the prothrombin consumption index of haemophilic plasma is shown in Table VII. Once again no significant difference is apparent in all six normals.

TABLE VII

EFFECT ON THE P.C.I. OF HAEMOPHILIC PLASMA OF ADDITION OF PLASMA FROM SIX NORMALS

Percentage Plasma Added	Prothrombin Consumption Index (%)					
	Male (1)	Male (2)	Male (3)	Male (4)	Female (1)	Female (2)
Nil	177					
0.25	12	15	41	18	14	53
0.5	<5	12	32	11	47	19
1.0	<5	<5	<5	16	<5	<5
100.0	11	<5	<5	9	5	25

Haemophilia with Normal Coagulation Time.—It was found that cases of bleeding diathesis resembling classical haemophilia in practically all respects but having

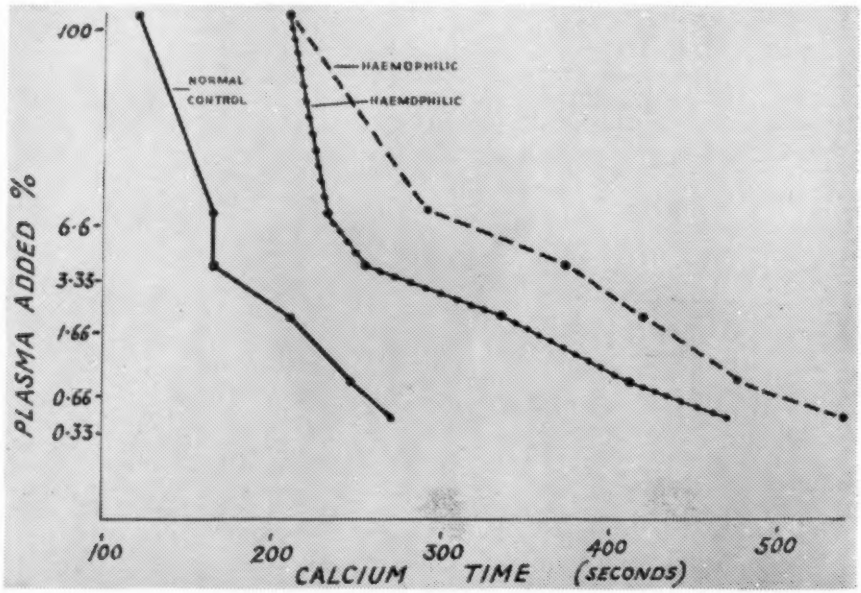

FIG. 9.—Shortening of the calcium time of known haemophilic blood produced by plasma of one normal control and two haemophilics with normal coagulation time. The percentage of plasma added is plotted on a logarithmic scale. The calcium time of the haemophilic plasma alone was 765 seconds.

a normal or near normal coagulation time were able to correct haemophilic blood to some extent but not nearly to the extent possessed by normal blood. A typical example incorporating two such cases (patients 44 and 47 in Table V) and one normal control is shown in Fig. 9.

Discussion

Coagulation Time of Whole Blood.—The results of this series confirm the observation that the coagulation time in haemophilia is often greatly prolonged. There appears to be a gradation of clotting times from those which are greatly prolonged to those which are only slightly, and some which are not at all, prolonged. Provided that only technically perfect venepunctures were considered and no blood transfusion had been given immediately before the test, it was found that in general the coagulation time in any one patient remained of the same order over long periods of time. In one patient, for instance, a Lee and White time of 150 minutes was found a year later to be 52 minutes: both these figures would be regarded as being greatly prolonged and therefore of the same order. Often the readings for the coagulation time did not vary by more than 20% over a year or more. With one exception, no patient having a coagulation time of 15 minutes or less on one occasion recorded a time in excess of 20 minutes on any other occasion. In some of these cases observations were available over a number of years. Even in the one exception the discrepancy was small. Much of course depends on the venepuncture, and unless this is efficiently performed misleading results may be obtained.

The capillary blood coagulation time as measured by the Dale and Laidlaw technique also proved reasonably satisfactory. In three children when only capillary blood was available, as adequate venous blood could not be obtained, it was the only available method. The Lee and White method is preferable and should always be done where possible; if veins are poor, or if repeated tests need to be done to evaluate the effect of therapy or for any other reason, valuable information can be obtained by the use of the Dale and Laidlaw method. It is important that the needle used be sharp and that the first drop of blood issuing be used, as the coagulation time shortens with the second drop (Gibbs, 1924; Christie, 1927). Furthermore the test tends to give false short rather than false long times, so that in all cases where a normal result is obtained it ought to be repeated on at least one occasion.

Quick's test for haemophilia was only occasionally helpful. Both normal and haemophilic blood are sensitive to the removal of platelets, and occasionally haemophilic blood may have even a greater degree of sensitivity. If the calcium time is initially prolonged, high speed centrifuging may increase the calcium time by many minutes, but the proportionate increase might still be no greater than that produced in normal blood. The important feature would then only be the initially prolonged calcium time. Finally, the test is non-specific, and other causes of a prolonged coagulation time, such as circulating anticoagulants, may give a "positive" result (Chargaff and West, 1946; Dreskin and Rosenthal, 1950). The test can only play a small part in the diagnosis of haemophilia.

Prothrombin Consumption Index.—The prothrombin consumption defect of haemophilia is amply demonstrated in this series of cases. It will be seen that by the use of serum from whole clotted venous blood abnormal prothrombin consumption

was found in all but three patients. In haemophilia with prolonged coagulation time the P.C.I. is almost invariably over 100%, but as the coagulation time shortens so the P.C.I. diminishes and its value may fall under 100%. In cases with a Lee and White coagulation time of 15 minutes or less where the diagnosis of haemophilia might be in doubt, the P.C.I. was of definite help in the diagnosis in all but three cases. It is unfortunately not in itself diagnostic because prothrombin consumption defects occur also in thrombocytopenic states (Soulier, 1948; Quick, Shanberge, and Stefanini, 1949; Merskey, 1950a; and others). Even a "functional platelet deficiency" has been described with normal number of platelets and abnormal prothrombin consumption (Soulier, 1948), and I have encountered similar cases. Nevertheless it still remains an important laboratory observation.

The two other methods of measuring the P.C.I. are less sensitive and offer very little advantage over the method using whole clotted venous blood. There is perhaps a case to be made out for the use of capillary blood in that, if an abnormal result is obtained, the diagnosis of haemophilia is extremely probable (Soulier, 1948). An abnormal result was obtained with this test only in one case where the Lee and White time was under 34 minutes; here the Dale and Laidlaw time was nine minutes. In other words, the P.C.I. of capillary blood is only likely to be abnormal if the case is one of haemophilia with prolonged coagulation time, and in this case the Dale and Laidlaw coagulation time should be abnormal. If venous blood is unavailable then it may be a useful confirmatory test in the diagnosis of haemophilia. It is also of interest in that it demonstrates that the tissue fluid of certain haemophilics seems to contain something which is absent in other haemophilics and this may account for some of the clinical differences observed.

Correction of Haemophilic by Normal Blood.—Patek and Stetson (1936) showed that there was a substance present in normal plasma and markedly deficient or unavailable in haemophilic plasma which in relatively small proportions effectively shortened the clotting time of haemophilic blood. This observation has been used as a diagnostic test for haemophilia by many authors. Furthermore it has recently been shown that a small proportion of normal blood can correct the prothrombin consumption defect of haemophilic blood (Quick, 1947; Soulier, 1948; Alexander and de Vries, 1949; and Merskey, 1950a).

In the present paper the test has been placed on a rough quantitative basis in an effort to "assay" the degree by which the test case and the normal control could correct these defects. Normal plasma can be added to the plasma of the patient and the effect on the calcium time of the latter recorded. Alternatively the blood or plasma of the patient can be added to that of a known haemophilic and the effect of this compared with that produced by a normal control.

Addition of Normal to Patient's Plasma.—If the calcium time of the patient is long, the shortening produced by the addition of small proportions of normal plasma may be very suggestive of a diagnosis of haemophilia. If the calcium time of the patient is only slightly prolonged the test has less scope since one soon reaches normal limits. In order to be completely certain in cases with a normal coagulation time it may be necessary to obtain known haemophilic blood and to perform the test in the reverse manner by testing the effect of the patient's blood on the known haemophilic blood.

Addition of Patient's to Known Haemophilic Blood.—It is easier to use citrate plasma rather than whole blood for this test, and the shortening of the calcium time seems to be at least as informative as the improvement in the P.C.I. Apparently no difference exists between donors of either sex and most people seem to be suitable as controls. This confirms the observations of Alexander and Landwehr (1948).

This test has been used as the final criterion in deciding whether or not to classify a doubtful case of haemophilia as such or whether to discard it from the group of true haemophilia. A number of suspect cases were thus excluded, and it was interesting to observe that the clinical picture of these was significantly different. The blood of patients with a circulating anticoagulant may fail to shorten the coagulation time of haemophilic blood but will not react to additions of normal blood and will, in fact, lengthen its clotting time when added in a small proportion. The difficulty of the test is to obtain haemophilic blood. It may perhaps be possible in the future to use haemophilic dog's plasma since the coagulation time of this is shortened by normal dog's plasma or by the use of a fraction prepared from human plasma (Graham, Buckwalter, Hartley, and Brinkhous, 1949), but no canine haemophilic plasma was available for trial.

It was of great interest to note the amount of normal blood required to correct the defects of haemophilic blood. Whereas under 1% of normal blood had a marked influence on these defects, 10% or even more was required for full correction of the coagulation time and prothrombin consumption index. When citrated plasma was used the prothrombin consumption index was restored to normal on the addition of under 1% of normal plasma, but the calcium time practically never reverted to normal even if over 10% of normal plasma was used. There appears to be a great difference between an improvement in these defects and full correction of them. Furthermore, very much more whole (uncitrated) blood seems to be required to bring the prothrombin consumption index back to normal than when citrated plasma is used. Very small proportions of whole blood do not seem to correct the coagulation defects of haemophilia.

Grades of Haemophilia.—From the evidence presented so far it seemed justifiable to grade the defect in the various haemophilics of the series. They appeared to fall roughly into three grades (Fig. 10).

Grade A : Lee and White Coagulation Time of Over 15 Minutes.—In this grade practically all the tests for haemophilia were positive. By a combination of a prolonged Dale and Laidlaw coagulation time and abnormal prothrombin consumption of capillary blood a reasonably certain diagnosis of haemophilia could be reached on finger-prick blood alone.

Grade B : Lee and White Coagulation Time of Between 10 and 15 Minutes.—In these patients laboratory tests showed fewer positive results. In particular, capillary blood usually consumed prothrombin during coagulation, while the figures for the prothrombin consumption index of venous blood tended to be lower than those recorded in Grade A.

Grade C : Lee and White Coagulation Time Under 10 Minutes.—In these patients laboratory tests showed still fewer positive results. In no case could abnormal prothrombin consumption on capillary blood be demonstrated. In 25% of these cases too even prothrombin consumption of venous blood became normal.

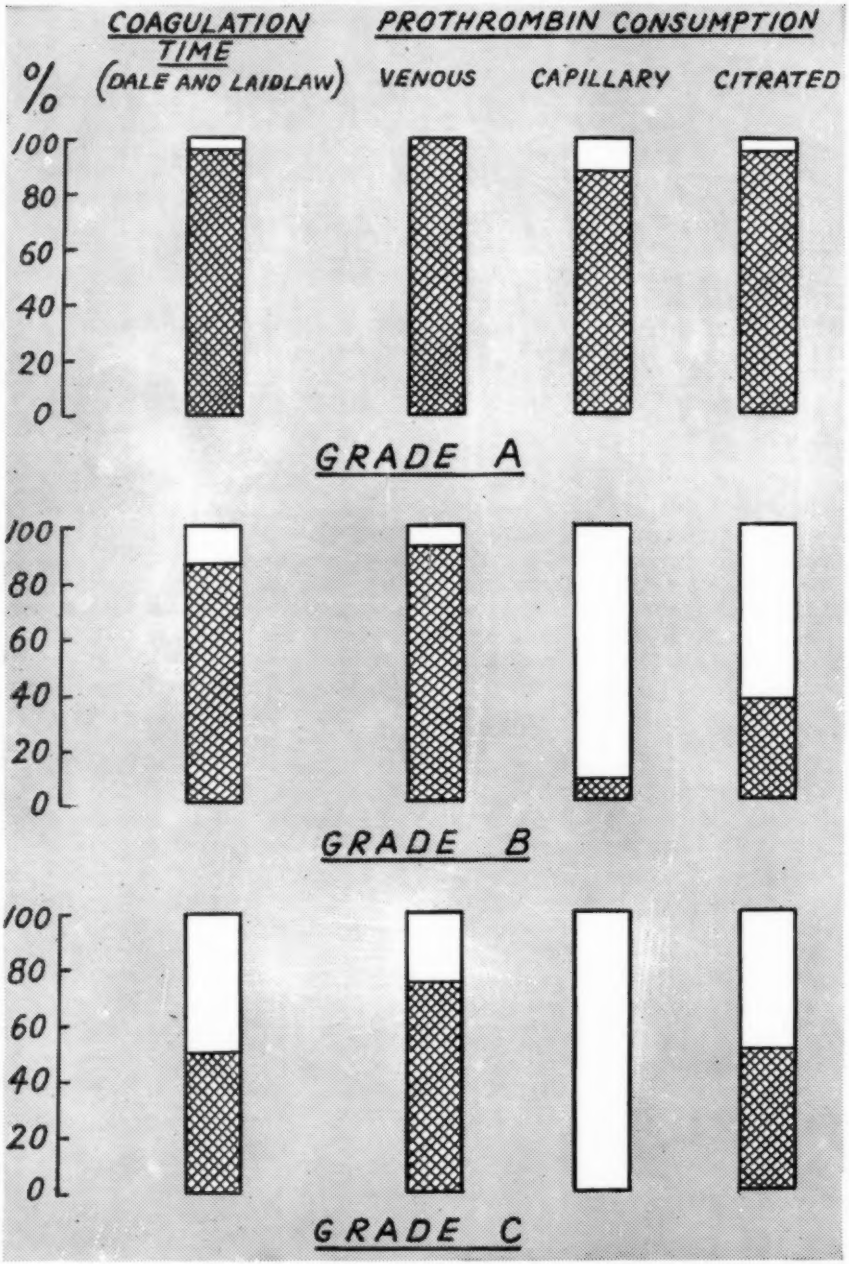

FIG. 10.—The "grades" of haemophilia based on Lee and White coagulation times, and the percentage of abnormal results found by other tests in patients of this series. *Grade A*, Lee and White time > 15 minutes. *Grade B*, Lee and White time 10–15 minutes. *Grade C*, Lee and White time < 10 minutes.

Fig. 10 demonstrates the percentage of positive results with the Dale and Laidlaw coagulation time and the three methods of measuring prothrombin consumption in the three grades of haemophilia. The degree of defect remained constant in any one individual and in any particular family (Table V) even over a number of generations.

While these three grades of the condition are described, the overall impression gained from this series was that all grades of the disease exist and one grade slides imperceptibly into the next.

It is even possible that the step from normal subjects to the milder grades of haemophilia may not be very large, but no evidence is at present available on this point. Finally it will be of great interest to observe the safety or otherwise of surgical procedures in the varying grades of this condition.

Correlation of Laboratory Findings and Clinical Picture.—The clinical picture was almost invariably severe when the Lee and White coagulation time was over 15 minutes (Grade A). It did not seem to matter by how much this figure was exceeded. But under 15 minutes (Grade B) the clinical picture seemed, in some cases, to be a little milder, and once the Lee and White time fell into the normal range (Grade C) a mild clinical syndrome was the rule rather than the exception. Of the 11 families whose Lee and White coagulation time was less than 15 minutes, seven had a mild clinical syndrome, one was intermediate, and three were very severe. In the seven families with the milder syndrome haemorrhages tended to be much less frequent and less severe, and complications, especially haemarthroses, slight or absent. Many of the patients were able to lead normal lives and were only slightly incapacitated. Yet even in these patients operations were poorly tolerated ; they bled excessively after dental extractions, and practically all of them had had at least one severe haemorrhagic episode seeming to endanger life. Of the three families with a normal coagulation time and a severe clinical syndrome, two patients had had almost every known complication of haemophilia and were severely incapacitated, and two members of another family had died of apparently spontaneous gastro-intestinal haemorrhage. Nine of these families are described in detail in other papers (Merskey, 1950b, c).

Summary and Conclusions

An analysis has been made of the value of the diagnostic procedures used in haemophilia. The series consisted of 72 patients. In 39% of these it was impossible to trace a family history of haemorrhagic disease.

While the lengthening of the coagulation time remained a very valuable diagnostic sign, a normal coagulation time by no means excluded the condition.

The Lee and White method of measuring the coagulation time was adequate provided technically imperfect procedures were rejected. The Dale and Laidlaw technique of measuring coagulation time was a little less reliable, but by no means valueless, and much information was obtained from its use. In three children only capillary blood was available and the test was then invaluable.

Quick's test for haemophilia was found to be of small diagnostic value only.

The measurement of the prothrombin consumption defect was a useful confirmatory test of haemophilia. The greatest information was obtained if this was measured on the serum from whole clotted venous blood. Serum from capillary

blood was only of value in cases with prolonged coagulation time. Serum from recalcified citrate plasma was intermediate in value between these two.

In doubtful cases, titration of the amount of antihaemophilic globulin in the plasma was of great value in establishing a diagnosis.

By the use of all these tests three grades of haemophilia were distinguished. The grades were not very clearly defined and intermediate cases existed. This gradation was reflected in both the clinical and the laboratory manifestations of the disease.

The degree of the defect in any one case seemed to remain constant over long periods of time.

The degree of the defect was constant in any family even over a number of generations.

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References

Abrahams, D. G., and Glynn, L. E. (1949). *Clin. Sci.*, **8**, 171.
Alexander, B., and de Vries, A. (1949). *Blood*, **4**, 752.
—— and Landwehr, G. (1948). *J. clin. Invest.*, **27**, 98.
Biggs, R., and Macmillan, R. L. (1948). *J. clin. Path.*, **1**, 269.
Birch, C. L. (1937). *Hemophilia*. Univ. Illinois Press, Urbana.
Brinkhous, K. M. (1939). *Amer. J. med. Sci.*, **198**, 509.
Chargaff, E., and West, R. (1946). *J. biol. Chem.*, **166**, 189.
Christie, R. V. (1927). *Quart. J. Med.*, **20**, 471.
Conley, C. L., Hartmann, R. C., and Morse, W. I. (1949). *J. clin. Invest.*, **28**, 340.
Dale, H. H., and Laidlaw, P. P. (1911–12). *J. Path. Bact.*, **16**, 351.
Dreskin, O. H., and Rosenthal, N. (1950). *Blood*, **5**, 46.
Gibbs, O. S. (1924). *Quart. J. Med.*, **17**, 312.
Graham, J. B., Buckwalter, J. A., Hartley, L. J., and Brinkhous, K. M. (1949). *J. exp. Med.*, **90**, 97.
Gram, H. C. (1920). *Bull. Johns Hopk. Hosp.*, **31**, 364.
Lee, R. I., and White, P. D. (1913). *Amer. J. med. Sci.*, **145**, 495.
Merskey, C. (1950a). *J. clin. Path.*, **3**, 130.
—— (1950b). In the press.
—— (1950c). In the press.
Patek, A. J., and Stetson, R. P. (1936). *J. clin. Invest.*, **15**, 531.
Quick, A. J. (1941). *Amer. J. med. Sci.*, **201**, 469.
—— (1947). Ibid., **214**, 272.
—— Honorato, C. R., and Stefanini, M. (1948). *Blood*, **3**, 1120.
—— Shanberge, J. N., and Stefanini, M. (1949). *Amer. J. med. Sci.*, **217**, 198.
Soulier, J. P. (1948). *Rev. Hémat.*, **3**, 302.

J. clin. Path. (1950), 3, 321.

THE COMMON CAUSES OF LYMPHOPENIA

BY

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Since differential blood counts are now reported in absolute numbers as well as in percentages, lymphopenia is frequently found and its significance questioned. Whitby and Britton (1946) say that the lymphocytes in the peripheral blood may be diminished in the acute stages of an infection, in conditions of exhaustion, after excessive *x*-ray irradiation, with vitamin deficiency, and in the terminal phase of uraemia. Wintrobe (1946) says:

> " Rarely, leucopaenia may be due to a reduction in the lymphocytes. This has been described in miliary tuberculosis and in instances of excessive exposure to irradiation. The action of certain chemical agents is much more pronounced on lymphocytes than granulocytes."

Wintrobe also regards a marked absolute reduction of lymphocytes as an unfavourable prognostic sign. These authors refer to the lymphopenia which is found in such haematological disorders as Banti's syndrome and agranulocytosis. To obtain a clearer picture of the common causes of lymphopenia differential leucocyte counts performed in one laboratory over a year were analysed. This laboratory serves a general hospital admitting all types of cases (babies and adults) except accidents, infectious fevers, and maternity cases. A few out-patients are also investigated at the laboratory. The children's department is small in relation to the rest of the hospital.

The blood counts were performed with the usual care and precautions taken in a hospital laboratory.

The lower limit of normal for a lymphocytic count is usually stated to be 1,500 per c.mm. (Wintrobe, 1939). However, it was decided to study only those cases in which the reduction was marked, so that, for the purpose of this paper, only lymphocyte counts of 1,000 per c.mm. or less have been classed as lymphopenic. The lowest count of all was one of 270 in a patient treated with *x* rays. Some patients with lymphopenia also had eosinopenia, but this finding was not constant.

A total of 647 patients had differential leucocyte counts performed on one or more occasions. Of this number 87 patients showed a lymphopenia at some time or other. The youngest was a boy of 8 years, an epileptic on tridione therapy, and the eldest a woman of 83 who was said to be suffering from pericolitis. The laboratory findings, diagnoses, and clinical notes of the 87 cases with a lymphopenia of 1,000 c.mm. or less at any point in the illness are set out in Tables I–VIII. The cases are grouped according to diagnosis.

TABLE III

GROUP III: TWELVE CASES OF RESPIRATORY DISEASE (EXCLUDING TUBERCULOSIS)

Case	Age	Sex	Hb (g.%)	Total W.B.C. (c.mm.)	Lymphocytes (c.mm.)	Neutrophils (c.mm.)	Eosinophils (c.mm.)	Monocytes (c.mm.)	Basophils (c.mm.)	Plasmocytes (c.mm.)	Diagnosis	Notes
33	48	F	14.0	22,000	880	20,240	—	880	—	—	Lobar pneumonia	Subsequent empyema; recovered
34	73	F	—	10,000	800	8,600	—	600	—	—	,, ,,	Died
35	62	M	13.0	6,000	480	5,280	—	240	—	—	Bronchopneumonia	,,
36	28	F	15.2	7,000	700	5,950	70	280	—	—	Lobar pneumonia	Recovered; profuse growth of pneumococci in sputum
37	34	F	12.2	8,000	960	6,240	160	480	—	160	,, ,,	Not severely ill; recovered
38	78	M	15.6	10,000	900	8,900	—	200	—	—	,, ,,	Recovered
39	77	F	14.8	16,000	800	14,880	—	320	—	—	,, ,,	,,
40	56	M	—	9,000	990	7,470	—	540	—	—	Chronic bronchitis and emphysema	Admitted with exacerbation; recovered
41	46	M	14.2	6,000	960	4,800	—	120	120	—	,, ,, ,,	,, ,, ,,
42	62	M	10.6	10,000	600	8,900	—	400	100	—	,, ,, ,,	Exacerbation with small pleural effusion; recovered
43	17	F	13.0	5,000	900	3,450	—	650	—	—	Pleural effusion	Recovered. (May have been tuberculous, although included in this group)
44	60	M	8.6	11,000	880	9,020	110	990	—	—	Carcinoma bronchus	Pleural effusion; hypochromic anaemia

TABLE IV

GROUP IV: TEN CASES OF IATROGENIC AND OCCUPATIONAL DISEASE

Case	Age	Sex	Hb (g.%)	Total W.B.C. (c.mm.)	Lymphocytes (c.mm.)	Neutrophils (c.mm.)	Eosinophils (c.mm.)	Monocytes (c.mm.)	Basophils (c.mm.)	Plasmocytes (c.mm.)	Diagnosis	Notes
45	65	F	7.7	7,000	980	5,600	—	420	—	—	Carcinoma bronchus	Nitrogen mustard therapy; pre-treatment lymphocytes normal; hypochromic anaemia
46	18	M	9.9	4,000	560	3,320	40	80	—	—	Lymphosarcoma	" " "
47	42	F	5.4	3,200	480	1,952	480	224	32	32	Carcinoma ovary	" " "
48	69	M	13.0	6,000	720	4,860	60	300	60	—	Carcinoma bronchus	Nitrogen mustard therapy; out-patient; pre-treatment lymphocytes not known
49	55	F	13.8	3,900	975	2,535	117	195	78	—	Carcinoma cervix	Treated with radium 9 years previously
50	?	F	12.9	3,350	938	2,278	—	67	—	67	*X*-ray worker	Routine check-up
51	70	F	4.15	27,000	270	13,500 (2,700 of these myelocytes)	—	—	—	—	Myeloid leukaemia	Following *x*-ray therapy (13,230 normoblasts included in total W.B.C.)
52	65	F	11.6	700	672	14	—	—	—	14	Agranulocytosis	Following "soneryl"; recovered
53	8	M	—	6,000	960	4,740	180	120	—	—	Epileptic	Tridione therapy; clinically satisfactory
54	15	M	11.2	47,000	470	42,535 (4,700 of these myelocytes)	1,410	940	1,410	—	Chronic myeloid leukaemia	Urethane therapy (235 myeloblasts included in total W.B.C.)

TABLE V

GROUP V: NINE CASES OF BLOOD DISEASE

Case	Age	Sex	Hb (g.%)	Total W.B.C. (c.mm.)	Lymphocytes (c.mm.)	Neutrophils (c.mm.)	Eosinophils (c.mm.)	Monocytes (c.mm.)	Basophils (c.mm.)	Plasmocytes (c.mm.)	Diagnosis	Notes
55	69	M	6.4	14,000	700	4,960 (2,240 of these myelocytes)	—	280	—	—	Chronic myeloid leukaemia (untreated)	(3,720 myeloblasts and 4,340 normoblasts included in total W.B.C.)
56	46	M	9.8	1,500	900	495	—	105	—	—	Aleukaemic myeloid leukaemia (untreated)	Myeloblastic crisis and died after 8 months in aleukaemic phase
57	48	M	5.45	8,000	960	6,400	—	400	240	—	Pernicious anaemia (untreated)	Complicated by pneumonia and cardiac failure; responded to liver with complete recovery
58	46	M	9.2	5,000	950	3,450	500	100	—	—	,, ,, ,,	Responded uneventfully to liver
59	76	M	4.0	2,700	810	1,782	27	81	—	—	Hypochromic anaemia (untreated)	Cause of anaemia not found; responded to iron
60	32	F	8.6	6,000	960	4,740	120	120	60	—	,, ,, ,,	Out-patient; further details not available
61	66	F	10.8	1,000	800	140	20	40	—	—	Diabetes and leucopenia	Clinical condition good; leucopenia persisted after diabetes controlled; cause not found
62	19	M	7.6	3,700	999	2,479	37	185	—	—	Splenic anaemia (untreated)	Haematemesis; general condition remains good
63	28	F	14.2	5,000	850	3,550	300	300	—	—	Acholuric jaundice (post-splenectomy)	Lymphocytes normal before splenectomy

TABLE VI

GROUPS VI AND VII: FIVE CASES EACH OF TUBERCULOSIS AND LIVER DISEASE

Case	Age	Sex	Hb (g.%)	Total W.B.C. (c.mm.)	Lymphocytes (c.mm.)	Neutrophils (c.mm.)	Eosinophils (c.mm.	Monocytes (c.mm.)	Basophils (c.mm.)	Plasmocytes (c.mm.)	Diagnosis	Notes
64	59	M	13.3	6,000	300	5,640	—	60	—	—	Tuberculous broncho-pneumonia	Died 12 days later
65	52	M	4.75	5,000	800	4,100	—	100	—	—	Pulmonary tuberculosis	Numerous haemoptyses; died 20 days later
66	45	M	—	13,000	910	11,700	—	390	—	—	Severe sacro-iliac tuberculosis	Went home against advice and lost sight of
67	52	M	9.2	3,000	510	2,460	—	30	—	—	Pulmonary tuberculosis	Died 5 days later
68	25	M	—	6,000	600	5,220	60	60	—	—	Miliary tuberculosis	Died 2 months later
69	52	F	12.2	3,000	870	1,680	150	210	60	30	Infective hepatitis	Lymphopenia subsided as condition recovered
70	50	F	14.8	7,000	910	5,950	—	140	—	—	Obstructive jaundice; gall-stones	Recovered spontaneously; refused operation
71	69	F	14.5	9,000	630	8,100	90	180	—	—	Metastatic carcinoma of liver	Radical mastectomy 4 years previously, admitted with ascites: necropsy showed liver extensively invaded with metastases
72	68	M	4.75	3,000	660	2,100	60	180	—	—	Cirrhosis of liver	Haematemesis; recovered temporarily, but died 1 year later
73	14	M	11.8	2,850	740	2,024	—	86	—	—	,, ,, ,,	Ascites for some weeks. Died 1 month later

TABLE VII

GROUPS VIII, IX, AND X: THREE CASES OF RETENTION OF URINE; TWO PSYCHOGENIC CASES; TWO CASES OF LYMPHADENOPATHY

Case	Age	Sex	Hb (g.%)	Total W.B.C. (c.mm.)	Lymphocytes (c.mm.)	Neutrophils (c.mm.)	Eosinophils (c.mm.)	Monocytes (c.mm.)	Basophils (c.mm.)	Plasmocytes (c.mm.)	Diagnosis	Notes
74	66	F	14.7	16,000	960	14,720	—	320	—	—	Cerebral thrombosis	Retention of urine; blood urea 68 mg.%: recovered
75	68	M	12.1	13,000	650	11,960	130	260	—	—	Prostatic hypertrophy	Retention of urine; blood urea 285 mg.%: discharged with a permanent suprapubic drainage
76	45	M	—	7,000	980	5,390	210	350	70	—	Disseminated sclerosis	Transient retention of urine with cystitis; subsided after treatment and discharged ambulant
77	23	F	14.8	4,000	1,000	2,840	—	80	—	80	Hysterical amnesia	No organic disease found
78	16	M	15.8	6,000	540	5,400	—	60	—	—	Anxiety state	Extensive investigation, but no organic disease found
79	52	F	8.9	11,000	660	9,900	110	330	—	—	Histiocytic medullary reticulosis (untreated)	Died within 7 days
80	43	M	12.2	2,800	560	2,072	—	168	—	—	Hodgkin's disease (untreated)	Died after a few months

TABLE VIII

SEVEN MISCELLANEOUS CASES

Case	Age	Sex	Hb (g.%)	Total W.B.C. (c.mm.)	Lymphocytes (c.mm.)	Neutrophils (c.mm.)	Eosinophils (c.mm.)	Monocytes (c.mm.)	Basophils (c.mm.)	Plasmocytes (c.mm.)	Diagnosis	Notes
81	17	F	5.0	500	455	10	—	—	—	15	Necrotic ileitis with *Bact. coli* septicaemia	Died on the day following admission. Cause of ileitis not found. 20 normoblasts included in total W.B.C.
82	23	M	—	20,000	1,000	18,000	—	1.000	—	—	Submaxillary abscess	Not severely ill; complete recovery with incision and sulphonamides
83	70	F	13.2	4,000	960	2,880	80	80	—	—	Cerebral thrombosis	Bedridden with hemiplegia for 3 weeks, slowly deteriorating
84	62	F	5.9	2,250	540	1,620	—	90	—	—	Sore tongue	Hypochromic anaemia, further clinical details not available (out-patient)
85	83	F	—	11,000	770	9,570	—	660	—	—	? Pericolitis	Further clinical details not available (out-patient)
86	53	F	14.2	2,900	841	1,972	29	58	—	—	Ulcerous stomatitis	" " " "
87	64	F	13.3	4,000	920	2,960	40	80	—	—	" Diarrhoea "	" " " "

Discussion

It appears that, although lymphopenia is sometimes associated with a poor prognosis, it is by no means invariably so. The commonest condition in which it occurred was cardiac failure, but this may be due partly to the high proportion of elderly patients with cardiac disease among the admissions to this hospital. Altana and Pulino (1947) have described the leucocyte picture in cases of heart disease, excluding as far as possible those cases complicated by chest or other infections. They express their counts as percentages only, but from their data the absolute values can be calculated, and it is then found that of 20 patients with well-compensated heart disease none showed lymphocyte counts of 1,000 or less; of 30 patients with a slight degree of congestion there were five with lymphopenia (17%); of 55 patients with severe congestion there were 17 with lymphopenia (31%); of 38 patients with left-sided cardiac failure and minimal venous congestion there were six with lymphopenia (16%); and of seven congenital malformations of the heart there were three with lymphopenia. In cases in the present series with pleural effusion repeated counts did seem to suggest that lymphopenia fluctuated with the presence of fluid in the chest, but in cases with cirrhosis of the liver it did not parallel the clinical condition. Case 72 had a normal lymphocyte count when almost moribund. In most of the cases in Groups II and III the prognosis was good, and in fact the only cases where lymphopenia gave a pointer to an ominous prognosis were those in Group VI. In other groups the prognosis depended on the primary disease.

The occurrence of lymphopenia in the lymphadenopathies has been noted by Robb-Smith (1947). The mechanism in these and in the blood diseases is presumably linked with the metabolic abnormalities of these disorders. The mechanism of its appearance in other conditions is more obscure and may be due to lymph stasis or the diminished production of lymphocytes. Wada (1940), having studied the lymphopenia of patients with uraemia and cholaemia, produced the condition experimentally in rabbits by inducing uraemia or cholaemia and related the blood findings to histological changes in the lymph nodes. He also found that removal of the rabbits' spleens exaggerated the lymphocyte depression. Another approach to some of the cases is provided by Selye's alarm reaction (1946) in which, during the phase of counter-shock, there is hypertrophy of the adrenal cortex and involution of lymphoid tissue. Reinhardt and Li (1945) have shown that administration of pituitary adrenotrophic hormone leads to a diminution of the lymphocytes entering the blood from the thoracic duct. Yoffey, Reiss, and Baxter (1946) have made further observations along the same lines.

Summary

Of 647 patients on whom routine blood counts were performed, 87 showed a lymphopenia of 1,000 per c.mm. or less at one time or another. The conditions with which this was associated have been analysed and the findings tabulated.

My thanks are due to my colleagues for making their case histories freely available to me, to Dr. C. Groves for valuable advice, and to Mrs. I. Gledhill for help in the collection of the records.

References

Altana, G., and Pulino, C. (1947). *Folia cardiol., Milano*, **6**, 193.
Reinhardt, W. O., and Li, C. H. (1945). *Science*, **101**, 360.
Robb-Smith, A. H. T. (1947). In *Recent Advances in Clinical Pathology*, ed. by Dyke, S. C., London.
Selye, H. (1946). *J. clin. Endocrinol.*, **6**, 117.
Wada, S. (1940). *Arb. med. Fak. Okayama*, **6**, 387.
Whitby, L. E. H., and Britton, C. J. C. (1946). *Disorders of the Blood*. London.
Wintrobe, M. M. (1939). *Bull. N. Y. Acad. Med.*, **15**, 223.
—— (1946). *Clinical Hematology*, 2nd ed. London.
Yoffey, J. M., Reiss, M., and Baxter, J. S. (1946). *Nature, Lond.*, **157**, 368.

J. clin. Path. (1950), 3, 332.

THE PLASMA VISCOSITY

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The viscosity of the plasma was studied briefly by Fahraeus in his original investigations (1921) into the blood sedimentation rate. In 1940 further studies were made by T'Ang and Wang, who concluded that it gave a more accurate picture of the activity of disease processes than the erythrocyte sedimentation rate. The plasma viscosity technique was independently developed by Whittington and his colleagues, who reached a similar conclusion (Miller and Whittington, 1942; Houston, Harkness, and Whittington, 1946).

In the present work an attempt has been made to study the importance of individual protein fractions in plasma viscosity changes and to assess the value of such a fractionation in diagnosis, prognosis, and the control of therapy.

Material

In all, viscosity measurements have been made in some 700 samples of plasma, and the complete fractional viscosity method has been studied in 292 samples, 47 from healthy subjects and the remainder from a wide variety of diseases. These include the following:

Rheumatoid arthritis	166	samples
Neurosis	64	"
Tuberculosis of lungs	49	"
" of pleura	22	"
" of lymph glands	8	"
" of kidneys	3	"
" of meninges	1	"
" of peritoneum	1	"
Rheumatic fever	38	"
Chronic rheumatic heart disease	33	"
Pneumonia	26	"
Disc degeneration	27	"
Disc prolapse	8	"
Fibrositis	24	"
Osteoarthritis	22	"
Malignant disease	19	"
Peptic ulcer	20	"
Gout	17	"
Ulcerative colitis	15	"
Malaria	13	"
Pulmonary fibrosis	13	"
Spondylitis ankylopoietica	12	"

Abscesses and carbuncles	11	samples
Hepatitis (acute and subacute)	9	,,
Bronchitis	9	,,
Bronchiectasis	7	,,
Traumatic disorders	7	,,
Tertiary syphilis	6	,,
Asthma	5	,,
Hypertension	6	,,
Coronary thrombosis	5	,,
Nephritis	4	,,
Urticaria and angio-neurotic oedema	4	,,
Amoebiasis	4	,,
Miscellaneous disorders	108	,,

Method

Fractionation.—Using minimal stasis, 10 ml. of blood was collected in a syringe previously rinsed with 3.8% sodium citrate. The blood was placed in a tube containing 0.1 ml. of double strength oxalate mixture,* well mixed, and then centrifuged to separate the plasma. The viscosity of the oxalate plasma was estimated. Calcium chloride (in this instance 0.03 ml. of 40% calcium chloride) was then added to precipitate the oxalate and induce clotting. (An excess must be avoided, as it maintains the fibrin in solution.) Half an hour is required for clotting at 37° C., or the sample may be left overnight at room temperature. The serum was obtained by expressing the clot and its viscosity estimated. A 4 *M*-ammonium sulphate solution was then added in a proportion of one volume of this to two volumes of plasma. In this way a portion of the serum globulin was precipitated. This is identical with the fraction separated by Jager and Nickerson (1948) in their method for estimating the γ globulin and shown to consist almost entirely of this protein. The protein so precipitated was then removed by filtration, using a Whatman 40 or 50 paper, and the viscosity of the filtrate estimated. A further portion of the globulin was then removed by the addition of one volume of 4 *M*-ammonium sulphate to three volumes of the filtrate, thus giving a 2 molar solution—i.e., approximately half-saturated. After filtration through a No. 50 Whatman paper the viscosity of this second filtrate was then estimated. If any of the precipitate came through in the early stages of filtration the liquor was returned to the filter.

Measurement of Viscosity.—Only the capillary type of viscometer is suitable for use with human plasma. This consists essentially of two reservoirs at different heights connected by a capillary tube. In a viscometer for clinical use there are three points that require consideration: (1) The instrument should use a reasonable amount of plasma. This must be a compromise, as a large capacity is associated with smaller errors and is easier to use but is less practicable for routine clinical use on account of its demands in plasma. It is above all important that the instrument be so designed that there is no wastage of plasma. (2) The accuracy should not be greatly affected by small errors of filling so that the instrument must be washed and dried between each of a series of tests. (3) It should be capable of being immersed in water so that it may be maintained at a constant temperature.

The Hess viscometer, which was designed for use with whole blood, cannot be inserted into a water bath and so lacks the precision which is essential for use with plasma.

The Ostwald viscometer is suitable, but as generally made is wasteful of plasma and thus as a rule requires too large an amount. The Ostwald viscometer used in the

* Ammonium oxalate 6 g. Potassium oxalate 4 g.; water to 100 ml. kept at 37° C. Tubes should not be dried in a hot oven.

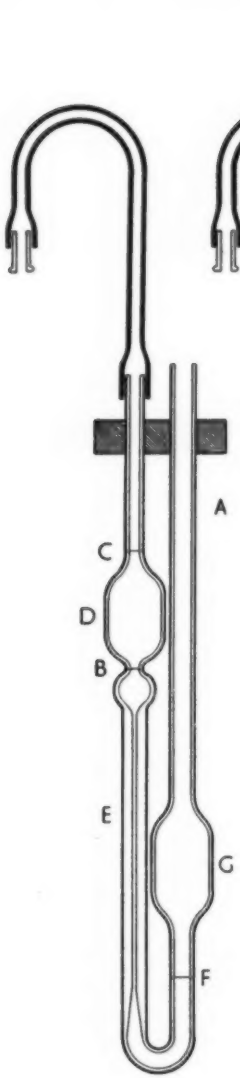

FIG. 1*a*
Ostwald viscometer

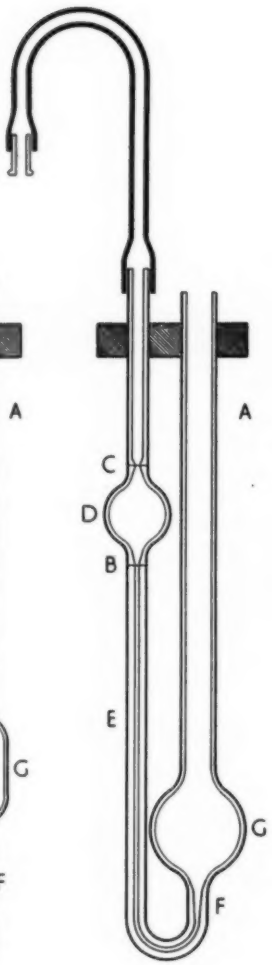

FIG. 1*b*
Modified Ostwald viscometer

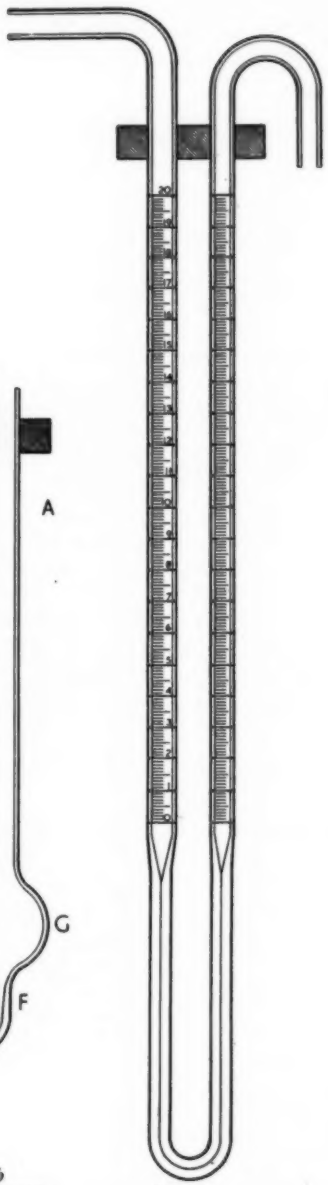

FIG. 1*c*
Whittington viscometer

earlier experiments is shown in Fig. 1*a*.* It requires at least 6.5 ml. of plasma, so that 20 ml. of blood is required for the test, but it is simple to use and accurate. It will be observed that an appreciable amount of plasma is required to fill the tube at the U-bend between the lower end of the capillary and the mark F; this is not used in the viscosity test and is thus wasted.

A smaller instrument made to the author's design is shown in Fig. 1*b*.† It requires 2.5 ml., but this volume of plasma is almost entirely used for the test, as the capillary tubing is continuous from one bulb to the other round the U-bend. Slight errors of filling are neutralized by the wide diameter of the lower bulb, which avoids an appreciable alteration in the head of pressure from over- or under-filling. Failure to dry the instrument before filling, for example, results in an error of only 0.2%. Thus, provided samples of similar viscosity are being tested, it is not necessary to wash and dry the instrument between tests. It should, however, always be washed after a high viscosity sample has been tested. Serum should never be placed in the instrument after plasma without washing, as clotting will occur. Similarly, samples containing ammonium sulphate should not be put into the instrument without washing if it has just contained serum or plasma, or serum containing a lesser saturation of ammonium sulphate, as precipitation occurs on the wall of the capillary and invalidates the result.

The Whittington viscometer (Fig. 1*c*) requires only 0.7 ml. of plasma, but the capillary is very fine, so that the instrument is difficult to wash and error may result from small particles of suspended matter. Owing to the small diameter of the right bend, or receiving limb, small errors of filling affect the head of pressure appreciably and must be corrected. The instrument must be washed and dried between each sample, so that much time is lost.

Temperature.—The temperature used in viscometry has unfortunately not been standardized. Most workers have used a temperature of 25° C. as in the experiments here set forth. Whittington and his colleagues have adopted a 20° C. standard. This is more readily maintained but less convenient in hot climates or summer weather, unless special equipment is available for cooling. It has, however, a theoretical advantage, as the viscosity of water is approximately 1 centipoise at 20° C. so that the relative viscosity is numerically identical with the value in centipoises. This, however, is not of great importance, as, owing to the effect of varying instrument design on the kinematic viscosity of protein solutions, results cannot at present be expressed in finite units. An approximate value for the timing at 20° C. may be obtained by adding 12.5% to the time required at 25° C. It is of course essential to mention the temperature used when expressing results, and it would be advisable that a standard temperature be adopted by all workers.

Calibration of the Viscometer.—If results are to be comparable with those of other workers the viscometer must be calibrated against two liquids of different viscosity. One of these may be water, but the other must be a protein solution similar to plasma. Calibration with crystalloid solutions or pure liquids is of no value if a viscometer is to be used for testing protein solutions (Harkness and Whittington, 1947). Table I gives the viscosity of solutions of pure human albumin, using the author's instrument (0_1). The viscosity is here calculated according to the formula:

$V = \frac{T_p}{T_w}$ where V = the viscosity

T_p = the time taken with the albumin solution

T_w = the time taken with water

* Supplied by Messrs. Gallenkamp, London.

† Supplied by the Manchester Scientific Glassware Co., 12, Wright Street, Manchester 15.

TABLE I

VISCOSITY OF PURE HUMAN ALBUMIN (LEDERLE) WITH VISCOMETER 0_1

Albumin (%)	Viscosity
25	6.01
20	3.35
15	2.12
10	1.54
6	1.29
5	1.24
4	1.18
3	1.13
2	1.08
0	1.00

(Water = 100)

To calibrate another instrument against viscometer 0_1 the time required for water and, for example, a 10% albumin solution is determined. The value for two constants A and B is then calculated according to the formula $V=AT+\frac{B}{T}$. For example, if the time taken for water is 34.6 seconds and for 10% albumin 56.0 seconds with the viscometer to be calibrated, two equations are obtained.

$$1.00=A\times 34.6+\frac{B}{34.6}$$

and $$1.54=A\times 56.0+\frac{B}{56.0}$$

If these two equations are solved the result obtained is

$$A=0.0263$$

and $$B=2.8$$

Substituting these in the formula $V=AT+\frac{B}{T}$ we have $V=0.0263\times T+\frac{2.8}{T}$ from which the viscosity of any sample of plasma or serum may be calculated by substituting for T the time taken with the unknown sample. It should be clearly understood that this gives the results in terms of the viscometer 0_1 and that these need not correspond with the figures obtained if a different viscometer is used as a standard. The sole advantage of standardizing to 0_1 is that the figures for normal values of the fractional viscosity given in this paper may then be used.

As an alternative to the above calculation a calibration chart may be prepared by plotting viscosity against time using the same formula.

Method of Using the Ostwald Viscometer

The viscometer is suspended in a tank holding preferably one to four gallons of water thermostatically controlled to within $\pm 0.3^\circ$ C. To carry out the tests an exact amount of plasma appropriate to the instrument—i.e., the amount used when calibrating it—is introduced into the limb A (Fig. 1*b*) by means of a fine-pointed pipette. If the plasma is run in carefully down the wall of the viscometer limb the formation of bubbles is avoided. After allowing three minutes for the temperature to become stabilized the plasma is sucked up to mark C. It now fills the bulb D and the capillary tube E. A small amount remains in the lower bulb G. The fluid is released and is then timed

with a stop watch till the mark B is reached. This time, which should be accurate to 0.1 seconds, is then substituted for T in the formula $V = AT + \frac{B}{T}$, or a calibration chart as already described may be used. On completion of the test the viscometer is dried on the outside and emptied by blowing gently through the mouthpiece. It is washed out first with 2.5% sodium hydroxide, then with water.

Calculation

As already described, figures were obtained for the plasma, serum, and two filtrates, and the viscosity differences due to fibrinogen, two fractions of globulin, and the albumin can then be estimated. The following example illustrates the method of calculating the relative viscosity:

Plasma	Serum	First Filtrate	Second Filtrate
1.54	1.40	1.42	1.48

(Viscosity of distilled water = 1.0)

The viscosity difference due to fibrinogen may be obtained by deducting the serum from the plasma viscosity. In order to avoid negative values for the other two fractions a deduction must first be made from the viscosity of the first and second filtrate for the contained ammonium sulphate. The viscosity of the ammonium sulphate solutions of the concentrations present in the filtrates were found to be 1.16 and 1.27 respectively. Thus the viscosity differences due to the ammonium sulphate were 0.16 and 0.27, and the corrected viscosity of the four samples is:

Plasma	Serum	First Filtrate less Ammonium Sulphate	Second Filtrate less Ammonium Sulphate
1.54	1.40	1.26	1.21

The fibrinogen viscosity difference therefore = 1.54 – 1.40 = 0.14 ; the globulin I viscosity difference = 1.40 – 1.26 = 0.14 ; the globulin II viscosity difference = 1.26 – 1.21 = 0.05 ; and the albumin viscosity difference = 1.48 – 1.27 = 0.21. For convenience the fractional viscosity difference will throughout the remainder of this paper be expressed as viscosity of water = 100, so that the results of the above fractionation would be fibrinogen, 14 ; globulin I, 14 ; globulin II, 5 ; albumin, 21. The total plasma viscosity will also be expressed as a viscosity difference, the viscosity of the contained water being deducted, giving, in my example, a figure of 54 for plasma. This will be found to equal the sum of the fractional viscosity differences.

Experimental Error.—The error involved in the actual viscosity measurement is very small and on repeated estimation does not exceed ±0.4%. The procedures of clotting and filtration used in the differential viscosity technique introduce additional sources of error, but the discrepancy between duplicate estimations is only slightly greater than from the simple viscosity measurement. In eight sets of duplicate

estimations in which each sample of plasma was divided into two, and the precipitations and filtrations carried out on each separately, the maximum error in the viscosity of the filtrates was ±1.0%. The error which may be found when the viscosity differences are calculated is of course greater, involving as it does the combined effect of subtracting two readings to obtain a relatively small difference. In the eight sets of duplicate estimations studied the maximum errors were as follows:

Fibrinogen	Globulin I	Globulin II	Albumin
±2%	±5%	±25%	±6%

Small changes (up to 3 units) in the viscosity differences in serial estimations cannot therefore be considered significant.

As with all studies concerned with plasma proteins gross error may result from stasis induced during the collection of blood samples. For example, a sample collected during constriction of the arm at a pressure of 100 mm. of mercury may have a viscosity 8% higher than a sample obtained at a pressure of 50 mm., and stasis continued for one minute may result in a further increase of 8% in the viscosity. In this way the fibrinogen viscosity difference may be caused to vary by as much as 36%. It is recommended that a pressure of 50 mm. of mercury should not be exceeded in the cuff used to induce stasis and that blood be collected as soon as the cuff has been applied. The viscosity should preferably be estimated the same day, but no significant error results from leaving the plasma to stand till the following day. Delay till the third day may result in a 2% reduction of the viscosity and should therefore be avoided.

Effect of Ammonium Sulphate on Viscosity of Protein Solutions

There can be little doubt that the effect of ammonium sulphate on the viscosity of protein solutions is very much more complex than would be inferred from the simple method of calculation used. Chick (1914) found that addition of salt up to a concentration of 1% had the effect of reducing the viscosity of serum proteins. Above this concentration the addition of further salt was without effect. This reduction of viscosity, which may be termed the initial salting factor, has been determined on a number of normal and pathological samples of serum, and has been found to amount to a viscosity difference of 1 to 4 in normal samples with a range up to 9 in pathological samples. In practice, however, it was not found advantageous to include an additional stage in the fractional analysis to allow for this, as it gives little information of value.

It should be understood that no attempt has been made to estimate the actual viscosity of the fractions isolated. Normal values for the viscosity differences will be given below and these will be used for comparison with the values obtained in disease. The actual values for the viscosity of the fractions do in fact correspond with that of a pure protein solution in the case of the albumin fraction (Fig. 2), but the fibrinogen and first globulin viscosity differences are greater than the viscosity of corresponding amounts of pure fibrinogen or γ globulin. It is unlikely that this is due in the case of globulin I to the presence of small amounts of β and α globulin

in the precipitate, as the specific viscosity of globulin II is less than that of globulin I (as measured by comparing the viscosity difference and the weight of the precipitate). The effect of the ammonium sulphate accounts for a part of this discrepancy but by no means the whole. A possible explanation of the remainder may lie in the theory postulated by Harkness and Whittington (1947) of a combined protein molecule of globulin and albumin having a viscosity coefficient greater than either. The viscosity differences could, if desired, be converted into actual viscosity values of corresponding solutions of the pure protein by reference to the data in Fig. 2, but such a conversion would be of purely academic interest and of no value for routine clinical purposes. Fig. 2 could also be used to convert viscosity differences into protein concentrations of fibrinogen, γ globulin, and albumin. As the second globulin fraction contains α, β, and γ globulins in varying proportions no relationship between viscosity and concentration is to be expected here. The "albumin" fraction (which also contains a certain amount of α globulin) shows a closer relationship than globulin II but is less accurate than fibrinogen or globulin I in the lower values. It is, however, preferable to retain the viscosity differences as such and not to attempt a conversion. The chief value

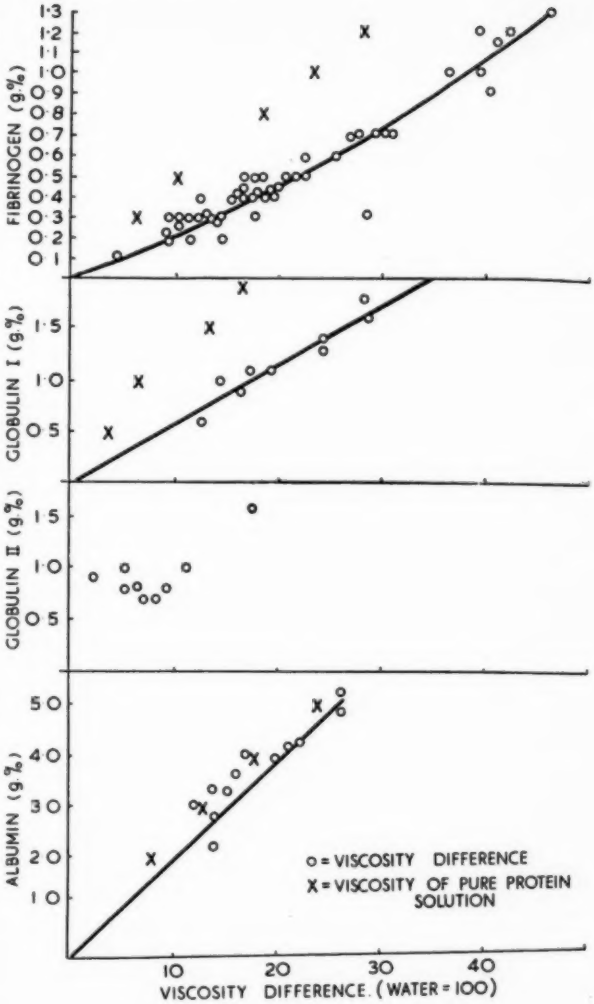

FIG. 2

of the method is to show relative changes and not absolute values, and these have in practice proved of great value in the detection and assessment of disease processes.

Results

Normal values were studied in 57 apparently healthy persons (mostly blood donors). Of these 36 were males and 21 females. No significant differences were found between the sexes, nor did age affect the values. The range of "normal" values encountered was as follows:

	Plasma	Fibrinogen	Globulin I	Globulin II	Albumin
Mean	58	13	18	6	21
Maximum	71	19	26	10	27
Minimum	44	5	12	2	17
Standard deviation ..	10	2	6	2	6

A brief summary of the results to be expected in disease has already been given elsewhere (Lawrence, 1949). It was pointed out that changes in the viscosity fractions were largely non-specific, being present in all processes involving tissue damage whether inflammatory, toxic, ischaemic, or due to trauma or other physical agents, and that the relative changes in the fractions depended more on the stage of the disease process than on its nature. Thus in acute disorders the fibrinogen and sometimes the second globulin fraction were raised during the first week, the first globulin fraction being unaffected till the second or third week. During recovery the changes were found to subside in the same order, so that the first globulin fraction often remained raised for several weeks after the disease process had ceased to be active. The albumin fraction was often reduced during the early stages and returned to normal after a variable time. In subacute disorders the sequence was identical but changes took place more slowly, and in chronic disorders the fractions might remain constantly abnormal or show repeated cycles of the above sequence. These changes are illustrated by serial studies in a normal person in whom artificial fever was induced, and also in acute, subacute, and chronic disorders (Table II). The E.S.R. is also shown in order to demonstrate its failure in certain instances to reflect the changes in the plasma proteins and to indicate the presence of tissue damage. The changes following haemorrhage are also shown. Here the fractions are all reduced and the subsequent order of recovery is fibrinogen (during the first 24 hours), globulin II, albumin, and globulin I (after two weeks).

In only a few instances could the fractional viscosity changes be said to be in any way specific. These are shown in Table III. They have already been discussed in the previous paper (Lawrence, 1949).

Discussion

The chief value of the differential plasma viscosity is that it is a comparatively simple method of indicating changes in the plasma proteins, particularly the fibrinogen and the γ globulin. Though these changes are largely non-specific a knowledge of their presence is often of great value both in diagnosis and in assessing

TABLE II

SERIAL STUDIES OF THE DIFFERENTIAL PLASMA VISCOSITY

Case No.	Disease	Day of Disease	E.S.R. (mm.) 2 to 15	Viscosity Differences				
				Plasma	Fibrinogen	Globulin I	Globulin II	Albumin
				44 to 71	5 to 19	12 to 26	2 to 10	17 to 27
	Normal subject given T.A.B. vaccine (i.v.) before inoculation	0	3	52	9	18	6	19
		1	7	52	13	14	6	19
		2	13	59	15	19	6	19
		3	12	63	20	16	8	19
		4	10	54	15	19	3	17
		13	11	62	9	27	7	19
		18	7	57	15	15	—	—
1	Malaria — Febrile	17	74	76	21	28	8	19
	Malaria — Afebrile and symptom-free	28	39	72	17	32	5	20
	Malaria — Convalescent	41	4	65	14	20	—	—
2	Rheumatic fever with carditis:							
	High salicylate doses	6	4	84	30	26	—	—
	Afebrile, reduced salicylate dosage	21	55	100	33	35	17	15
	Heart clinically normal	41	13	72	21	23	—	—
	Convalescent	62	10	65	16	22	9	18
3	Rheumatic carditis following scarlet fever	15	2	92	28	34	14	16
	Still tachycardia and subpyrexia	21	86	115	49	39	11	16
	Tachycardia less (afebrile)	31	64	100	32	39	9	20
	Tachycardia still present	64	12	78	17	33	—	—
	Clinically recovered	100	7	65	13	23	—	—
4	Subacute lupus erythematosus	90	60	90	32	30	12	16
	Afebrile	130	27	79	20	35	5	19
	Still lassitude	160	20	71	14	30	—	—
	Symptom-free	190	—	73	15	33	4	21
		220	8	68	15	21	—	0
		Day of Study						
	Cell volume (%) 52	1	11	95	28	43	10	14
	Cell volume (%) 49	19	9	68	17	24	11	16
5	Rheumatic heart (mitral stenosis) Cell volume (%) 54	30	2	104	21	—	—	—
	Cell volume (%) 46	46	33	91	27	34	10	20
	Cell volume (%) 48	62	13	75	17	28	7	22
	Relapsed, rheumatic nodules Cell volume (%) 53	87	4	80	28	27	—	—
	Cell volume (%) 51	94	21	90	24	34	—	—
	Cell volume (%) 54	123	—	83	19	32	6	26
	Quiescent Cell volume (%) 50	169	—	67	14	25	—	—
6	Rheumatoid arthritis (before treatment)		14	79	23	29	10	23
	Receiving copper compound	28	9	72				
	Receiving copper compound	50	7	81	24	30	5	22
	Receiving copper compound	130	23	86	26	35	2	23

TABLE II—*Continued*

Case No.	Disease	Day of Study	E.S.R. (mm.) 2 to 15	Viscosity Differences				
				Plasma	Fibrinogen	Globulin I	Globulin II	Albumin
				44 to 71	5 to 19	12 to 26	2 to 10	17 to 27
6 (cont.)	Rest period	153	15	76	26	28	7	15
	On gold treatment ..	181	20	64	19	19	10	16
	On gold treatment ..	200	5	63	18	21	6	18
	On gold treatment ..	222	10	64	17	19	2	26
	On gold treatment ..	253	2	50	12	14	3	21
	On gold treatment maintenance dosage ..	313	5	59	14	18	6	21
7	Rheumatoid arthritis (before treatment) ..		6	66	21	23	9	13
	On gold treatment ..	43	7	71	22	23	6	20
	On gold treatment ..	78	3	66				
	On gold treatment ..	108	2	61	15	24	7	15
	On gold treatment ..	135	3	65	15	24	3	23
	On gold treatment maintenance dosage	255	5	70	15	22	7	26
8	Rheumatoid arthritis (before treatment) ..		9	68	24	19	7	18
	On gold treatment ..	42	19	68	23	24	6	15
	On gold treatment ..	70	8	67	17	12	17	21
	On gold treatment ..	110	10	67	21	24	5	17
	On gold treatment ..	144	15	60	19	17	8	16
	On gold treatment ..	172	15	60	12	18	8	22
	Gold discontinued ..	206	4	60	8	22	7	23
	Gold discontinued ..	297	4	62	16	20	5	21
9	Haematemesis (gastritis) Hb (%) 25	1	—	26	6	6	4	10
	Hb (%) 34	2		30	10	5	4	10
	Hb (%) 22	3		33	9	3	6	12
	Hb (%) 66	4		44	14	5	9	16

the results of therapy. Previously the E.S.R. has been largely used for this purpose, and recently the simple plasma viscosity, but each of these methods may give normal values in the presence of gross tissue damage and cannot therefore be relied on. Apart from disease of the brain, spinal cord, and meninges, which does not modify the plasma proteins, normal values for the differential plasma viscosity may occasionally be found in localized disease such as appendicitis, furuncle, or small malignant tumours if without metastases, but is never normal when widespread active disease is present. Space does not permit of a full discussion of this aspect, but an indication of the relative value of the E.S.R. and the differential and total plasma viscosity will be found in the previous paper.

The test is also of value in the diagnosis and assessment of blood loss, particularly where this has been occult as in gastric and duodenal haemorrhage. The rapid return of the fibrinogen viscosity level to normal when haemorrhage has ceased gives a ready indication of progress, and the slow recovery of the first globulin fraction gives useful confirmation of a belated diagnosis of occult haemorrhage.

In addition, specific changes indicated in Table III, the very high globulin I and fibrinogen in myelomatosis, the high globulin I in liver disease, and the low

TABLE III

POSSIBLE DIAGNOSTIC VALUE OF CERTAIN DIFFERENTIAL PLASMA VISCOSITIES

Case No.	Disease	Day of Disease	Normal E.S.R. 2–15	Plasma Viscosity				
				Fibrinogen	Globulin I	Globulin II	Albumin	Plasma
				5–18	12–25	2–10	17–27	44–71
	Myelomatosis and carcinomatosis of bone:							
1	Carcinomatosis of prostate		107	70	78	—	—	178
2	Carcinomatosis of prostate		112	25	38	—	—	98
3	Carcinomatosis of breast		47	63	40	17	20	140
4	Myelomatosis		122	140	405	—	—	570
	Lymph gland disease:							
5	Tuberculosis of mediastinal lymph glands	20	40	37	13	15	11	76
		40	22	25	25	12	15	77
		90	20	22	17	13	13	65
		120	5	19	16	5	21	61
		150	8	14	20	5	21	60
6	Hodgkin's disease	60	2	31	21	11	15	78
7	Lymphosarcoma	—	4	16	10	10	22	58
8	Chorion carcinoma of testis with extensive involvement of preaortic glands		20	30	19	—	—	78
	Liver disease:							
9	Subacute infective hepatitis (1)*	40	—	21	57	6	23	96
	Subacute infective hepatitis (2)	60	—	20	42	—	—	88
10	Infective hepatitis (convalescent)		13	18	25	12	24	77
11	Subacute necrosis of liver		7	15	26	—	—	68
12	Subacute necrosis of liver		32	16	24	8	18	64
13	Cirrhosis of liver			17	31	11	19	78
14	Multiple secondary deposits		37	12	26	12	14	64
	Renal disease:							
15	Chronic glomerulonephritis			17	10	—	—	55
16	Subacute glomerulonephritis		9	11	15	1	17	42
17	Acute glomerulonephritis		27	16	23	9	15	63
18	Lupus erythematosus kidney		28	16	12	5	9	42
19	Toxic nephritis (tertiary syphilis)		85	35	34	9	13	91
	Toxic nephritis (tertiary syphilis)		110	45	32	—	—	106
	Toxic nephritis (tertiary syphilis)		22	39	32	17	12	99
	Toxic nephritis (tertiary syphilis)			46	31	—	—	110

* Thymol turbidity (1) 9, (2) 9; colloidal gold (1) 4, (2) 5; Takata-Ara test (1) ++, (2) +.

value or lack of increase of this fraction in lymph gland disease may occasionally assist in diagnosis, but the information which the test gives as to the stage of disease is more often of value.

Summary

Technical aspects of the measurement of the plasma viscosity are discussed with particular reference to suitable types of instrument and their calibration for use with protein solutions.

A method for estimating the viscosity of individual protein fractions is described, and values found in 47 healthy individuals are noted.

From a study of 700 samples in a wide variety of diseases it is concluded that the differential plasma viscosity gives useful information as to changes in the plasma proteins, particularly the fibrinogen and γ globulin fractions, and that it is a more reliable indicator of these changes than the E.S.R. or the simple plasma viscosity. The changes in the differential viscosity at different stages of disease are noted and their non-specific nature stressed.

Possible diagnostic applications are suggested.

I wish to thank Professor R. B. Whittington of the University of Minnesota for his kind assistance and guidance in the intricate problems of viscometry, and Dr. J. H. Kellgren, Director of the Rheumatism Research Centre, Manchester University, and Professor S. L. Baker for their interest and advice.

References

Chick, H. (1914). *Biochem. J.*, **8**, 261.
Fåhraeus, R. (1921). *Acta med. scand.*, **55**, 1.
Harkness, J., and Whittington, R. B. (1947). *Biochim. biophys. Acta*, **1**, 487.
Houston, J., Harkness, J., and Whittington, R. B. (1946). *Acta tuberc. scand.*, **19**, 153.
Jager, B. V., and Nickerson, M. (1948). *J. clin. Invest.*, **27**, 231.
Lawrence, J. S. (1949). *Ann. rheum. Dis.*, **8**, 209.
Miller, A. K., and Whittington, R. B. (1942). *Lancet*, **2**, 510.
T'Ang, B. H. Y., and Wang, S. H. (1940). *Chin. med. J.*, **57**, 546.

J. clin. Path. (1950), 3, 345.

CREATINE AND GUANIDOACETIC ACID METABOLISM IN PITUITARY DISEASE

BY

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Schrire (1937) found that patients with an acidophil tumour of the pituitary excreted creatine in the urine, and this was later confirmed (Cumings, 1944). It was also shown in the latter paper that the fasting blood creatine was raised above the normal in acromegaly, and the results of a creatine tolerance test were given in five cases of pituitary disorders. The cause of the creatinaemia and creatinuria in acromegaly is obscure, but it had been shown previously that the urine excretion of creatine paralleled the height of the blood creatine provided that a level higher than 0.6 mg./100 ml. had been obtained. Guanidoacetic acid has been shown to be a precursor of creatine (Borsook and Dubnoff, 1940; 1941) and its estimation in pituitary disorders does not appear to have been recorded previously. This paper records a large series of patients with pituitary disorders, together with a few cases of thyrotoxicosis, in whom creatine and guanidoacetic acid studies have been made.

Material and Methods

Creatine studies have been made on 39 subjects consisting of 10 normal controls, 11 with acromegaly, nine with chromophobe adenomata or suprapituitary cysts, six with miscellaneous pituitary disorders, and three with thyrotoxicosis. The diagnosis was made in most cases at operation and by histology, or by clinical and radiological evidence which was unmistakable. Guanidoacetic acid was estimated in the urine in 26 out of the 39 subjects, and, in a few patients, more than one examination was made.

All patients were placed on a meat- and fish-free diet and 24-hourly urines were collected over a five- or six-day period. Creatine and creatinine were estimated according to the principles of the Folin and Wu technique (1919). A creatine tolerance test as described by Tierney and Peters (1943) was performed, usually on the third day, and the blood levels of creatine and creatinine estimated according to the technique of Peters (1942) as in my previous paper, but using a photoelectric colorimeter with a filter of 520 μ.

Urinary guanidoacetic acid was estimated as described by Hoberman (1947), following the technique of Dubnoff and Borsook (1941) as modified by Sims (1945),

after each urine had been treated with arginase to remove any arginine present. The arginase was prepared by the technique described by Hoberman (1947).

Results

Examples of results in each group are recorded in Appendix 1 and Appendix 2, and one example in each group is shown in the figures.

Normal Controls.—None of the 10 controls showed any creatinuria, and the amount of urinary creatinine was reasonably constant from day to day. The fasting blood creatinine was 0.5 mg./100 ml. or below in nine cases and in the other case 0.6 mg./100 ml., and this can be regarded as normal since Samuels, Sellers, and McCaulay (1946) obtained figures of up to 0.6 mg./100 ml. in normal controls even though Tierney and Peters (1943) found figures of up to 0.5 mg./100 ml. only. Guanidoacetic acid was estimated in four of these controls and a normal excretion of 30–50 mg. a day was obtained in all except one, a very large muscular man and a heavy manual worker, who excreted 60–80 mg. a day. The results found in this patient are shown in Fig. 1. It might be mentioned that not infrequently a little creatine is excreted in the first day of the test as a result of the creatine in the diet of the preceding day.

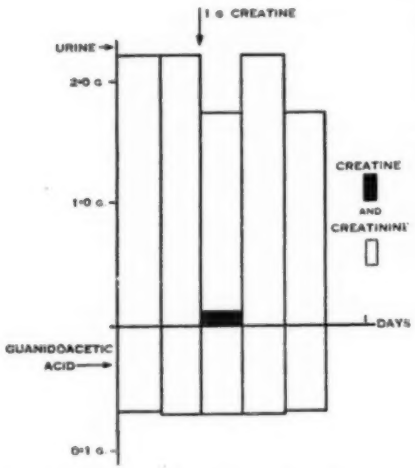

FIG. 1.—Diagram of guanidoacetic acid excretion in a normal control (N. H.).

Chromophobe or Suprapituitary Tumours.—The nine patients in this group showed results identical with the normal control group as regards urinary creatine and creatinine. The fasting blood creatine in all cases was within normal limits, but the rise in blood level was somewhat higher after 1 g. of creatine than was to be seen in the control group. Guanidoacetic acid was estimated in all except one case, and raised excretion rates were seen in all the patients except one, the only one who had a suprapituitary cyst. The usual excretion was as high as 80–100 mg. a day. The

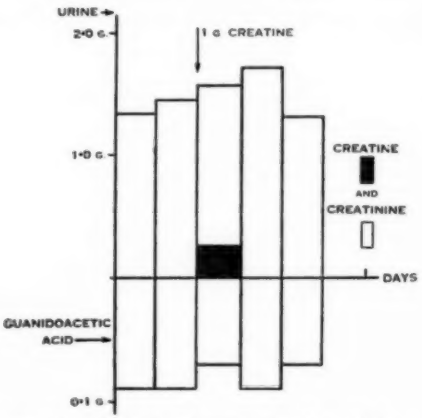

FIG. 2.—Diagram of guanidoacetic acid in a case of chromophobe adenoma.

patient with a raised blood creatinine had a severe nephritis from which he died post-operatively. Fig. 2 shows the findings in one case.

Acromegaly.—There was creatinuria in all except two patients, one of whom was examined nine years after removal of the pituitary acidophil tumour, and the other (S.D.) who was clinically normal following hormonal therapy. There was a raised fasting blood creatine in 10 out of the 11 patients, the only normal level of creatine being seen in the clinically normal patient who had received hormone treatment. The creatine tolerance test gave results similar to those previously recorded (Cumings, 1944).

Guanidoacetic acid was estimated in seven patients in this group, which included the patient apparently cured by hormones and another only examined post-operatively. These two patients showed a slightly raised excretion of guanidoacetic acid of up to about 60–80 mg. daily, but one of them excreted very little urine in each 24-hour period. The remaining five typical cases all showed a markedly increased excretory rate, usually of about 100 mg. daily.

A few of the patients were examined after operation and after hormone therapy as well as before these treatments. One patient, five years after operation, showed an improvement in the creatinaemia and creatinuria, and, except for one day during the test period, passed no more guanidoacetic acid in the urine than a normal control. Another patient nine years after operation showed no creatinuria even though the fasting blood creatine was slightly raised. One patient who showed the usual chemical findings seen in acromegaly was given hormone therapy, but after six months there were no improvements in blood or urine chemistry though there had been some clinical improvement. The other patient similarly treated, but for a much longer period, was practically normal when examined clinically and pathologically. An example of one of these cases is shown in Fig. 3.

Miscellaneous Pituitary Disorders.—This group consisted of two patients with tumours of mixed cell type (both acidophil and chromophobe cells), both of whom were diagnosed in the laboratory as having overactivity of acidophil cells; three patients with gross obesity and other evidence of pituitary dysfunction; and one very small, thin patient who also had a suprapituitary cyst. All these patients showed creatinuria, together with a raised fasting blood creatine. The very thin patient of the 39 patients examined showed the highest rise in blood creatine after ingestion of 1 g. of creatine by mouth. The guanidoacetic acid excretion of this patient one and a half years after operation was normal even though the

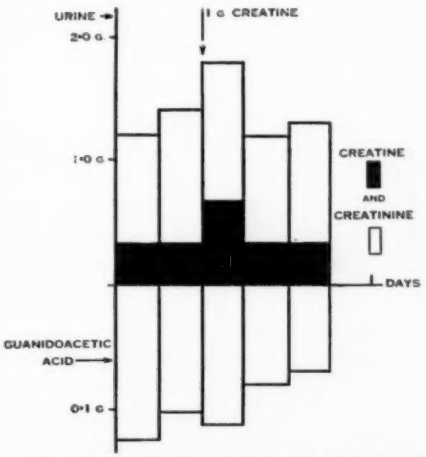

FIG. 3.—Diagram of guanidoacetic acid in a case of acidophil adenoma.

creatinuria still persisted. Guanidoacetic acid was also estimated in three other patients before operation. The obese patients so examined both showed a raised urinary output. One patient with a so-called "pituitary abscess" gave a normal rate of excretion of guanidoacetic acid.

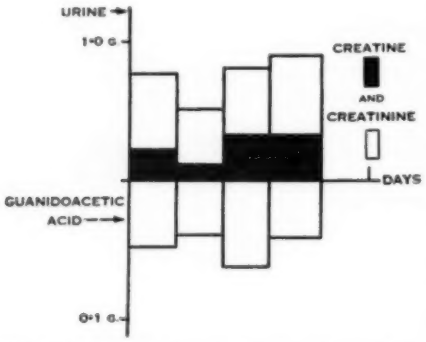

FIG. 4.—Diagram of guanidoacetic acid excretion in a case of thyrotoxicosis.

Thyrotoxicosis. — Three patients were examined and all showed creatinuria with a fasting blood creatine, although in no case was this latter figure as high as that seen in the cases of acromegaly. All three cases showed a normal excretion of guanidoacetic acid. The results found in a moderately severe case of thyrotoxicosis with a basal metabolic rate of +75% are shown in Fig. 4. Table I is a summary of the blood creatine curves of the cases recorded in Figs. 1–4.

TABLE I

BLOOD CREATINE CURVES AFTER 1 G. CREATINE IN FOUR CASES RECORDED IN THE DIAGRAMS

Condition	Time	Creatinine (mg./100 ml.)	Creatine (mg./100 ml.)
Normal	Fasting	0.9	0.3
	1 hour after creatine	1.25	0.55
	2½ ,, ,, ,,	1.0	0.5
Chromophobe adenoma ..	Fasting	0.7	0.5
	1 hour after creatine	0.9	0.9
	2½ ,, ,, ,,	0.8	0.7
Acromegaly	Fasting	0.62	0.88
	1 hour after creatine	0.75	1.75
	2½ ,, ,, ,,	0.75	0.85
Thyrotoxicosis	Fasting	0.7	0.9

Discussion

The results obtained in the examination of this large series of pituitary disorders confirm the earlier findings in 1944 as far as creatine metabolism is concerned, and there can be no doubt that in acromegaly with an acidophil tumour of the pituitary there is creatinaemia and creatinuria. It is seen that a large part of the ingested creatine is rapidly excreted in the urine and that the blood soon loses its increased quantity of creatine. Previously it had not been possible to distinguish

biochemically between a normal patient and a patient with a chromophobe adenoma, even though one could distinguish between these two and an acromegalic patient. It was also not possible by creatine metabolic studies to distinguish between thyrotoxicosis and acromegaly. From the results of the guanidoacetic acid studies, however, it is now possible to determine into which of these four groups any one patient falls. Table II shows the distinguishing biochemical features between the normal person and the three other conditions discussed.

TABLE II

DISTINGUISHING BIOCHEMICAL FEATURES OF THE NORMAL AND CERTAIN PITUITARY DISORDERS

Disease	Urine Creatine	Urine Guanidoacetic Acid	Fasting Blood Creatine
Acromegaly	++	++	Over 0.6 mg./100 ml.
Chromophobe tumour	0	+	Less than 0.6 mg./100 ml.
Thyrotoxicosis ..	++	Normal	Over 0.6 mg./100 ml.
Normal	0	,,	Less than 0.6 mg./100 ml.

Guanidoacetic acid is formed in the kidneys (Borsook and Dubnoff, 1941; Borsook, Dubnoff, Lilly, and Marriott, 1941) by transamidination from the amino-acids glycine and arginine, and is then transformed into creatine by methylation, probably utilizing methionine. This latter process probably takes place in the liver (Borsook and Dubnoff, 1940, 1947; Bloch and Schoenheimer, 1941), although there is some evidence from examinations made in cases of nephritis that this can also take place in the kidneys (Samuels *et al.*, 1946). It would appear, therefore, that the overaction of some of the pituitary cells affects these chains of events and so alters the urinary excretion of these substances.

There is in acromegaly not only an increased production of guanidoacetic acid but also an increased formation of creatine, hence the increase in production of the former substance must be quite considerable. It is different in a patient with a chromophobe adenoma as there is here an excess of guanidoacetic acid but no increase in creatine excretion; it would appear unlikely that any storage of creatine is taking place as this would need to be considerable, and, further, there is no increased excretion of creatinine over the normal, nor is there any creatinaemia. Equally, the excess of guanidoacetic acid must be an excess of production otherwise the blood level of creatine would drop and there would be a lowered excretion of creatinine. Further, because of the normal urinary creatinine and blood creatine it cannot be assumed that the guanidoacetic acid is not being made into creatine. It may be mentioned that in neither acidophil nor chromophobe tumours can any evidence be found biochemically of liver damage, as a number of liver function tests have been carried out in these patients and no abnormality has yet been detected; further, in one patient only was there any evidence of renal disease,

and the results obtained in that case, apart from the level of the blood creatinine, were not significantly different from the others in his group.

Borsook and Dubnoff (1947) showed that the presence of creatine in high concentration may retard the methylation of guanidoacetic acid to such an extent as to give a raised excretion of this substance. This cannot be the mechanism in acromegaly, for then one would expect to see the same process in thyrotoxicosis, but in this condition the guanidoacetic acid excretion is normal. It is therefore unlikely that the pituitary thyrotropic hormone plays any part in acromegaly, and further proof of this is seen in the inability of thiouracil to affect the creatinuria in acromegaly as it does in thyrotoxicosis (Schrire, 1948).

Hoberman, Sims, and Engstrom (1948) showed that methyl testosterone given to normal men produced creatinuria and also an increased urinary excretion of guanidoacetic acid, and they state that this is due to an increase in synthesis of creatine as a result of the effect of this hormone on the reactions involved in the synthesis of guanidoacetic acid. Samuels, Henschel, and Keys (1942) showed that if the methyl testosterone be given for some weeks then there is also creatinaemia and creatinuria occurring usually after 10 days' treatment with methyl testosterone. In the same way testosterone propionate as well as methyl testosterone produced similar results although not giving such a high level of excretion of creatine and guanidoacetic acid in the urine (Hoagland, Shank, and Gilder, 1944). These findings would seem to give a hint as to the mechanism causing the disturbance that is found in pituitary disease. It is not suggested that it is necessarily either of these two hormones that is responsible, but it does seem probable that the acidophil and the chromophobe cells each elaborate hormones that act in the manner suggested by Hoberman *et al.*, and it does seem to be true that the effects of both methyl testosterone and testosterone propionate are those seen in acromegaly. This would seem to be a basis for further study and one on which to build a programme of hormonal therapy for these two types of patients.

Summary

Creatine studies have been made in 10 normal controls, in 26 patients with pituitary disorders, and in three with thyrotoxicosis, and the urinary excretion of guanidoacetic acid followed in 26 of these 39 subjects. It has been shown to be possible biochemically to distinguish between the normal, the thyrotoxic, the acromegalic, and the patient with a chromophobe adenoma of the pituitary.

The possible causes of the biochemical abnormalities have been discussed and the possibilities of abnormal hormonal activity in the two pituitary disorders mentioned.

I wish to thank the many physicians and surgeons of the National Hospital, and also Dr. J. S. Richardson, for allowing me to conduct these investigations on their patients and for access to their case records; Drs. J. G. Greenfield and W. Blackwood for their histological opinions; and Mr. R. C. Shortman for his technical help. Lastly I should like to express my appreciation to Dr. E. A. H. Sims, of Yale University School of Medicine, who very kindly sent me a supply of guanidoacetic acid and also informed me of some of his work.

REFERENCES

Bloch, K., and Schoenheimer, R. (1941). *J. biol. Chem.*, **138**, 167.
Borsook, H., and Dubnoff, J. W. (1940). Ibid., **132**, 559.
—— —— (1941). Ibid., **138**, 389.
—— —— (1947). Ibid., **169**, 247.
—— —— Lilly, J. C., and Marriott, W. (1941). Ibid., **138**, 405.
Cumings, J. N. (1944). *Brain*, **67**, 265.
Dubnoff, J. W., and Borsook, H. (1941). *J. biol. Chem.*, **138**, 381.
Folin, O., and Wu, H. (1919). Ibid., **38**, 81.
Hoagland, C. L., Shank, R. E., and Gilder, H. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 49.
Hoberman, H. D. (1947). *J. biol. Chem.*, **167**, 721.
—— Sims, E. A. H., and Engstrom, W. W. (1948). Ibid., **173**, 111.
Peters, J. H. (1942). Ibid., **146**, 179.
Samuels, L. T., Henschel, A. F., and Keys, A. (1942). *J. clin. Endocrinol.*, **2**, 649.
—— Sellers, D. M., and McCaulay, C. J. (1946). Ibid., **6**, 655.
Schrire, I. (1937). *Quart. J. Med. n.s.*, **6**, 17.
—— (1948). *Clin. Sci.*, **7**, 49.
Sims, E. A. H. (1945). *J. biol. Chem.*, **158**, 239.
Tierney, N. A., and Peters, J. P. (1943). *J. clin. Invest.*, **22**, 595.

APPENDIX 1

In this appendix a representative protocol for one normal control and each type of case discussed is set out.

Normal (E. M.)

	Urine		
Day	Volume	Creatinine	Creatine	Guanidoacetic Acid
1	2,300 ml.	1.20 g.	0	0.035 g.
2	1,700 ,,	1.14 ,,	0	0.052 ,,
3*	1,920 ,,	1.12 ,,	0.31 g.	0.051 ,,
4	1,450 ,,	0.96 ,,	0	0.044 ,,
5	2,450 ,,	1.04 ,,	0	0.056 ,,

Creatine Tolerance Test

Fasting	0.5 mg./100 ml.	0.4 mg./100 ml.
1 hour after creatine	0.75 ,, ,, ,,	0.55 ,, ,, ,,
2½ hours ,, ,, ...	0.62 ,, ,, ,,	0.48 ,, ,, ,,

Chromophobe Adenoma.—A single woman of 25 complained of failing vision for seven years, and headache and hirsuties for six years. Menstruation was normal. Examination showed a coarse skin, which was greasy, and there was a definite deposit of hair over the face. There was a bitemporal lower quadrant defect with rather pale

*Creatine tolerance test (1 g. by mouth) on this day.

discs in the eyes. No other abnormality was found clinically or radiologically. Keto-steroid excretion, blood sugar curve, and C.S.F. were normal. At a later admission there was an additional hysterical factor also present. There was insufficient evidence of progression of the lesion causing the eye signs to warrant operative interference.

Urine

Day	Volume	Creatinine	Creatine	Guanidoacetic Acid
1	910 ml.	0.96 g.	0	0.075 g.
2	945 ,,	0.95 ,,	0	0.080 ,,
3*	1,250 ,,	0.94 ,,	0.13 g.	0.085 ,,
4	1,070 ,,	1.02 ,,	0	0.108 ,,
5	440 ,,	0.33 ,,	0	0.029 ,,

Creatine Tolerance Test

Fasting	0.9 mg./100 ml.	0.5 mg./100 ml.
1 hour after creatine	1.4 ,, ,, ,,	1.6 ,, ,, ,,
2½ hours ,, ,, ...	0.9 ,, ,, ,,	1.5 ,, ,, ,,

Acromegaly.—A woman of 53 with five children noticed enlargement of and pain in the hands for 17 years before admission. The feet enlarged four years later. A diagnosis of acromegaly was made eight years ago. Menstruation ceased five years ago. On examination there were typical acromegalic features. The eye fields were full, but there was a central scotoma in the right eye. The blood pressure was slightly raised, but no other abnormal physical signs were found. Radiologically the jaw was typically acromegalic and the pituitary fossa a little deep. Hormonal therapy was adopted because symptoms had become static.

Urine

Day	Volume	Creatinine	Creatine	Guanidoacetic Acid
1	2,200 ml.	1.58 g.	0.31 g.	0.075 g.
2	1,050 ,,	0.90 ,,	0.08 ,,	0.078 ,,
3*	3,208 ,,	2.16 ,,	0.12 ,,	0.107 ,,
4	2,010 ,,	1.41 ,,	0.20 ,,	0.097 ,,
5	2,050 ,,	1.44 ,,	0.21 ,,	0.109 ,,

Creatine Tolerance Test

Fasting	1.4 mg./100 ml.	1.35 mg./100 ml.
1 hour after creatine	2.0 ,, ,, ,,	1.5 ,, ,, ,,
2½ hours ,, ,, ...	1.5 ,, ,, ,,	1.75 ,, ,, ,,

Mixed "Dermoid" Tumour.—A man of 58 complained of failure of vision for four years and of impotence for two years. No headache or vomiting. Physical examination showed optic atrophy and perception of hand movements only on the right side, and on the left side a temporal hemianopia. The skin was smooth and of

* Creatine tolerance test (1 g. by mouth) on this day.

fine texture, and no axillary hair was present. The testes were small. Radiologically there was gross enlargement of the sella. Operation revealed a cystic mass in the pituitary, containing sebaceous-looking material. Histology showed this to be a "dermoid" tumour.

	Urine			
Day	Volume	Creatinine	Creatine	Guanidoacetic Acid
1	905 ml.	0.65 g.	0.08 g.	0.053 g.
2	910 ,,	0.78 ,,	0.07 ,,	0.065 ,,
3*	502 ,,	0.43 ,,	0.24 ,,	0.032 ,,
4	435 ,,	0.49 ,,	0.32 ,,	0.035 ,,
5	725 ,,	0.68 ,,	0.20 ,,	0.042 ,,

Creatine Tolerance Test

Fasting	0.9 mg/100 ml.	0.7 mg./100 ml.
1 hour after creatine	1.25 ,, ,, ,,	1.75 ,, ,, ,,
2½ hours ,, ,, ...	1.12 ,, ,, ,,	1.75 ,, ,, ,,

Obesity with Amenorrhoea.—A married woman of 35, whose third and last child was 8 years old, gave one year's history of increasing obesity, having gained 4 stone. Menstruation was almost absent. She was somewhat drowsy and there was increased sweating. There was no headache or visual symptoms. There was some temporal pallor of both optic discs, but no other abnormal physical signs were found. The B.M.R., blood count, C.S.F., and urinary ketosteroids were all normal. The blood sugar curve was normal; radiological examinations were also negative.

	Urine			
Day	Volume	Creatinine	Creatine	Guanidoacetic Acid
1	1,510 ml.	1.12 g.	0.24 g.	0.108 g.
2	1,660 ,,	1.24 ,,	0.35 ,,	0.136 ,,
3*	1,224 ,,	1.40 ,,	0.55 ,,	0.105 ,,
4	1,240 ,,	1.07 ,,	0.30 ,,	0.098 ,,
5	1,870 ,,	1.20 ,,	0.11 ,,	0.104 ,,

Creatine Tolerance Test

Fasting	0.8 mg./100 ml.	1.3 mg./100 ml.
1 hour after creatine	1.0 ,, ,, ,,	1.7 ,, ,, ,,
2½ hours ,, ,, ...	0.8 ,, ,, ,,	1.4 ,, ,, ,,

Thyrotoxicosis.—A single woman of 28 complained of mental and bodily fatigue for three years, and scanty periods for one year. There was also a complaint of proneness to ocular fatigue. Examination revealed no evidence of myasthenia or other

* Creatine tolerance test (1 g. by mouth) on this day.

neurological disorder. There was evidence of vasomotor instability. The pulse rate was between 80 and 90; the B.M.R. was slightly raised (+20%). Clinically she was considered to have a mild thyrotoxicosis, and under treatment she improved in hospital and after discharge.

Urine

Day	Volume	Creatinine	Creatine	Guanidoacetic Acid
1	1,900 ml.	1.42 g.	0.48 g.	0.047 g.
2	2,250 ,,	1.47 ,,	0.40 ,,	0.041 ,,
3*	2,530 ,,	1.73 ,,	0.21 ,,	0.055 ,,
4	1,190 ,,	0.95 ,,	0.42 ,,	0.061 ,,
5	1,700 ,,	1.32 ,,	0.23 ,,	0.065 ,,

Creatine Tolerance Test

Fasting	0.85 mg./100 ml.	0.65 mg./100 ml.
1 hour after creatine	1.25 ,, ,, ,,	0.77 ,, ,, ,,
2½ hours ,, ,, ...	1.3 ,, ,, ,,	0.65 ,, ,, ,,

* Creatine tolerance test (1 g. by mouth) on this day.

APPENDIX 2

This appendix contains a brief clinical abstract of the cases recorded in Figs. 2, 3, and 4.

Fig. 2 (13106/49. N.H.).—A woman of 32 with a history of amenorrhoea and headaches for 17 years, and poor vision for nine months.

Examination showed a well-built, normal-looking woman. There was primary optic atrophy and a complete temporal field loss on the right side only. There was also an early loss in the upper and outer part of the left field. The uterus and ovaries were small, but there were no other abnormal physical signs. A radiograph of the skull showed considerable enlargement of the pituitary fossa. Operation revealed a typical chromophobe adenoma (verified histologically). The tumour was removed and there was uneventful recovery.

Fig. 3 (7371/48. N.H.).—A woman of 42 whose sole complaint was of failing vision for 3½ months.

Examination showed a middle-aged, alert woman with unmistakable acromegalic features. There was a large head, massive maxillae, and slightly prognathous jaw. There was little soft tissue hypertrophy. Hands and feet were all large and broad. The skin was smooth and of a fine texture. In the eyes both fundi showed definite pallor of the discs, and some bitemporal scotomata were present. The blood pressure was raised, but no other abnormal physical signs were found. Radiologically the pituitary fossa was enlarged.

At operation a pituitary acidophil adenoma (confirmed histologically) was found and partially removed.

Fig. 4 (31707/49. W.H.).—A woman of 50 with a six months' history of tremor. There was said to be no loss in weight.

Examination revealed a middle-aged, rather thin woman with bilateral exophthalmos, with tremor of the outstretched hands, but no obvious sweating. The thyroid showed a diffuse soft enlargement. The pulse rate varied from 90 to 100 a minute, but the blood pressure was normal. B.M.R. +75%; blood count normal; blood cholesterol 197 mg. per 100 ml. Thiouracil produced considerable improvement in the clinical condition apart from the exophthalmos. There has been a gain in weight of 4 lb. to 8 st. 10½ lb.

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SOME EFFECTS OF NITRATES IN BACTERIOLOGICAL MEDIA

BY

G. T. COOK AND R. KNOX

From the Public Health Laboratory, Oxford

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In the routine examination of faeces by means of the "screening" plate previously described (Knox, 1949) it was found that on certain batches of the medium known gas-producing organisms failed to produce gas from the mannitol. Since this was the first time that the medium had failed in over a year's routine use it was felt that an explanation would be of some interest.

The explanation proved to be simple. It was recalled that a little earlier an error had been discovered in one stage of the bulk preparation of the laboratory tryptic digest broth; after digestion nitric acid had been used instead of the usual hydrochloric acid. At the time the possible importance of this was not appreciated, but when this error was revealed and related to the odd failure of gas production on the screening plates, the true explanation was immediately suggested. It is known that bacterial gas production is inhibited by the addition of hydrogen acceptors such as nitrate (Pakes and Jollyman, 1901) or tetrathionate (Knox, Gell, and Pollock, 1943) to cultures or washed suspensions of gas-producing organisms which are capable of using these substrates instead of oxygen as alternative hydrogen acceptors. The exact mechanism is not quite clear, but it would appear that the formation of hydrogenlyase is suppressed in a specific and probably competitive way, since it is only with organisms capable of reducing these substrates that gas production is inhibited in their presence. But whatever the precise explanation the following evidence shows that nitrate in the screening plates was in fact acting as a powerful suppressor of gas formation.

Suppressor Effects of Nitrate

1. Samples of the defective medium were found to contain nitrate. It was calculated that the concentration of nitrate in the medium would be of the order of 0.02M.

2. Batches of medium with and without potassium nitrate (0.02M) were prepared and inoculated in the usual way with salmonella and coliform organisms. The nitrate plates showed good acid production from mannitol but no gas; the control plates showed normal production of both acid and gas.

Other Effects of Nitrate

H_2S Production.—It had been observed that on the defective batches of medium, in addition to gas production from mannitol, production of H_2S was almost or

completely suppressed. This was also shown to be due to the nitrate in the medium: normal batches of medium A of the screening plate inoculated with H_2S producing strains of salmonellae showed excellent H_2S production which was completely suppressed in similar plates containing 0.02M potassium nitrate. That this effect may be of some practical importance in routine work was shown by some experiments with Wilson and Blair's medium made with and without 0.02M potassium nitrate. The control plates showed typical blackening with the characteristic halo and sheen around salmonella colonies, whereas the plates containing nitrate showed green colonies without any blackening. It is clearly possible that the unsuspected presence of other hydrogen acceptors could account for the failures which occur from time to time with Wilson and Blair's medium.

The explanation of this interference with H_2S production is obscure, but again it may be due to competition between organic or inorganic sulphur compounds on the one hand and nitrate on the other for a common enzyme system.

Effect on Blood Plates.—It was observed about this time that some batches of blood agar plates showed patchy brown discoloration usually in the neighbourhood of bacterial colonies. These areas were shown to be related to the presence of nitrate in the medium. Blood agar plates with and without 0.02M potassium nitrate were inoculated with each of the following five cultures: *Staphylococcus aureus, Staphylococcus albus, Corynebacterium diphtheriae gravis* and *mitis*, and a starch-fermenting gelatin-liquefying corynebacterium (Jebb, 1948). There was brown discoloration of the plate containing nitrate inoculated with each of the first four cultures, all nitrate-reducers. There was no discoloration of the nitrate plate inoculated with the last culture nor of any of the inoculated plates without nitrate. Starch-fermenting gelatin-liquefying corynebacteria are known to be unable to reduce nitrate, so it seemed as though the brown discoloration was related not to the presence of nitrate but to its reduction to nitrite. This suggestion was confirmed by finding that the addition of potassium nitrite to blood agar plates gave a similar brown discoloration. These observations formed the basis of the test for nitrate reduction described in the accompanying paper (Cook, page 359).

Discussion

These observations are of some interest since they illustrate admirably the intimate relation which can exist between routine and research in a bacteriological laboratory or, in the words of the late Dr. Marjory Stephenson, the different " levels " of microbiology, and the kinds of way in which each can be a stimulus to the other. A mistake in the media room led to a puzzling problem which was only solved by the use of information derived from fundamental studies of bacterial metabolism ; the application of this led in turn to a new test useful in routine work.

Summary

The accidental use of nitric instead of hydrochloric acid in routine medium making led to some interesting results, since the presence of nitrate in the basal medium accounted for (1) failure of gas production from mannitol by known gas-producing organisms ; (2) failure of H_2S production by known H_2S producers ; (3) failure of salmonella organisms to produce the characteristic blackening on Wilson

and Blair's medium ; and (4) a patchy brown discoloration on blood agar plates associated with oxidation of haemoglobin to methaemoglobin by nitrate-reducing organisms.

Our thanks are due to Mr. A. H. Tomlinson, M.A., for performing the chemical tests, and to Mr. A. Peacock for drawing attention to the accidental use of nitric acid in the medium.

References

Jebb, W. H. H. (1948). *J. Path. Bact.*, **60**, 403.
Knox, R. (1949). Ibid., **61**, 343.
—— Gell, P. G. H., and Pollock, M. R. (1943). *J. Hyg., Camb.*, **43**, 147.
Pakes, W. C. C., and Jollyman, W. H. (1901). *Proc. chem. Soc. Lond.*, **17**, 39.

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A PLATE TEST FOR NITRATE REDUCTION

BY

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From the Public Health Laboratory, Oxford

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Some effects of nitrates in bacteriological media are described in the accompanying paper (Cook and Knox, page 356). Among the observations made was that of a dark zone round colonies of nitrate-reducing organisms on blood agar containing potassium nitrate. Further experiments along these lines were carried out and resulted in the present plate test for nitrate reduction.

Method

The test is based on the oxidation of haemoglobin to methaemoglobin in the presence of nitrites and is carried out by making a stab inoculation into a blood agar medium containing potassium nitrate.

Potassium nitrate may be either (1) incorporated in the blood agar medium, or (2) added to it before use by means of a " nitrate strip."

1. A 20% solution of potassium nitrate (sterilized by steaming for one hour) is added to 6% horse blood tryptic digest heart agar to give a final concentration of 0.1% potassium nitrate. The plates are poured unlayered.

2. A blotting-paper strip containing potassium nitrate is placed on the centre of a layered 6% horse blood digest heart agar plate at the time of inoculation. The strips are prepared by soaking sheets of Ford's blotterettes (" Ford 428 Mill ") in a warm 40% solution of potassium nitrate. The sheets are hung up to dry in a 37° C. incubator and then cut up into small strips of approximately 16 × 10 mm. The strips are put into test-tubes and autoclaved for 10 minutes at 10 lb.

Two cultures can be conveniently tested on the same plate, each being stabbed into the centre of opposite halves of the medium. The results are read after 18–24 hours' incubation at 37° C., but plates which are negative should be reincubated for a further 24 hours. A positive result is indicated by a dark green-brown zone surrounding the site of inoculation and most easily seen against a white background or by transmitted light (Figs. 1, 2, and 3). The dark zone is due to the oxidation of haemoglobin within the red cell to methaemoglobin, the presence of which was confirmed by spectroscopic examination. This change is due to the gradual diffusion of nitrite produced by the organisms from potassium nitrate. The diameter of the zone varies with the time of incubation, depth of medium, and nature of the organism, but is usually between 2 and 4 cm. It is not advisable to inoculate more than two cultures on plates of approximately 9 cm. diameter as, with three or more positive strains, the extensive diffusion of nitrite may result in discoloration throughout the medium.

The plates are normally incubated aerobically, though better results with *Haemophilus* and *Pseudomonas* strains have been obtained on anaerobic incubation. Immediate inspection of the plate on removal from the jar shows the inoculated area surrounded by a zone of cherry red colour. On exposure to air this zone rapidly darkens, and after 15–20 minutes on the bench the colour has changed to the characteristic dark greenish-brown of methaemoglobin.

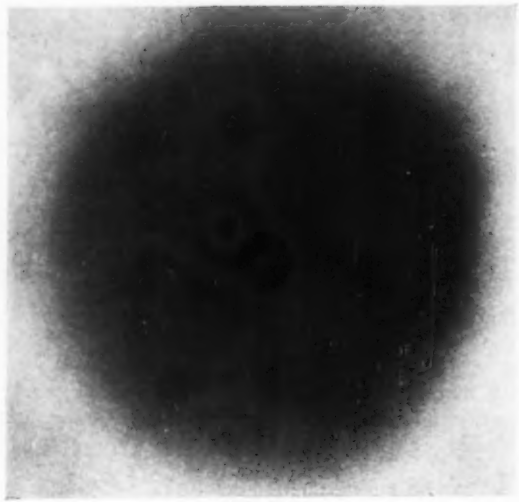

FIG. 1.

FIG. 3.

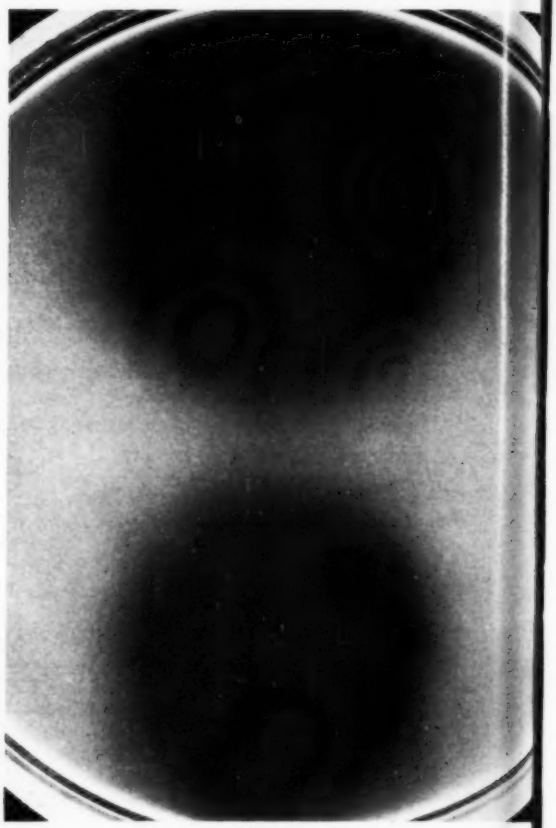

FIG. 2.

FIG. 1.—Stab inoculation of *Bact. coli* on 0.1% nitrate plate incubated for 24 hours.

FIG. 2.—Stab inoculation of *C. diphtheriae* var. *gravis* (above) and *C. diphtheriae* var. *intermedius* (below) on 0.1% nitrate plate incubated for 24 hours.

FIG. 3.—Stab inoculation of *Staph. aureus* (above) and a starch-fermenting gelatin-liquefying corynebacterium (below) on 0.1% nitrate plate incubated for 24 hours. The corynebacterium does not reduce nitrate.

Comparison of the Plate Test with Nitrate Broth

One hundred and seven strains from 13 genera have been tested for nitrate reduction by each of the two methods described in this paper and after growth in 0.1% nitrate broth. The plates were examined after 24 and 48 hours' aerobic incubation, and the broth cultures were tested for the presence of nitrite by the Griess-Ilosvay method (Wilson and Miles, 1946) after five days' incubation. Negative broth cultures were tested for the presence of unreduced nitrate by the addition of zinc powder (ZoBell, 1932).

Similar results were obtained with each of the three methods: 71 strains were nitrate reducers and 36 did not reduce nitrate. All but one of the 71 strains able to reduce nitrate gave a positive result by each of the plate methods after 24 hours. The exception, a diphtheroid, did so after 48 hours. Weak results by the plate methods were shown by some *Pseudomonas* and *Haemophilus* strains, though these strains always gave a satisfactory positive result on anaerobic incubation. In fact, each of the six strains of *H. influenzae* and *H. para-influenzae* included in the series gave a more definite result by the plate test carried out anaerobically than by the Griess-Ilosvay method.

Discussion

Certain points concerning the optimal concentration of nitrate in the medium and the method of inoculation are of special interest. It was found that, using potassium nitrate in a concentration of only 0.05% in the medium or of 10% in the strips, negative results were obtained with certain vigorous nitrate-reducing coliform and staphylococcal strains. Some of these strains also gave a negative Griess-Ilosvay test after 24 hours though positive at six hours, and the negative results on the plates may be due to rapid reduction of nitrite to substances which failed to discolour the medium. This difficulty was overcome by increasing the nitrate in the medium and the strips to 0.1% and 40% respectively and so ensuring a concentration of nitrite sufficient to produce a visible colour change in the cell haemoglobin.

It is also important that the method of inoculation of the plate should be a stab into the medium rather than a surface streak. Although most of the positive strains also reduced nitrate when inoculated on to the surface of the medium, some strains of *Haemophilus* and two of *Corynebacterium diphtheriae* var. *intermedius* failed to do so. Some degree of anaerobiosis appeared to be necessary for nitrate reduction by these organisms, all of which gave satisfactory results aerobically with a stab inoculation or with a surface inoculation provided the plate was incubated anaerobically. A positive result obtained by placing a " nitrate strip " across a streak inoculation of *C. diphtheriae* var. *intermedius* on a blood agar plate was shown to be due to the semi-anaerobic conditions produced under the strip.

It is well recognized that false negative results may be obtained when a nitrate broth culture is tested for the presence of nitrite by the Griess-Ilosvay method, as some species are able to reduce nitrites almost as rapidly as they are formed. A number of methods have been suggested in order to overcome this difficulty, and it is possible, for instance, to test for nitrite reduction products such as hydroxylamine, ammonia, or free nitrogen in the medium, though certain precautions have to be taken before reliable results are obtained (Conn, 1936). A more satisfactory and simple method is the zinc dust test for residual nitrate described by ZoBell

(1932), who recommends that it should invariably be made in conjunction with the test for nitrites when the latter is negative. One of the advantages of the plate test for nitrate reduction is that the cell haemoglobin acts as a sensitive and irreversible indicator of the presence of nitrites and so identifies as nitrate reducers organisms capable of removing nitrites in a fluid medium by further reduction. Of the 71 strains which gave a positive nitrate test on the plate, only 45 were positive by the Griess-Ilosvay technique. The remaining 26 cultures, however, were all negative to the zinc dust test.

The plate test is easy to carry out and the results can be read by simple inspection of the plate, in many cases within a few hours of inoculation. "Nitrate strips" kept on the bench maintain their activity for several weeks and can be employed with the ordinary horse blood digest agar plate. This is a point of some practical importance for the routine bacteriologist, who will probably seldom need to test for nitrate reduction. It is possible that a simpler method of performing the test may increase the usefulness of this procedure in diagnostic bacteriology. In this laboratory the rapidity of the plate test has proved of assistance in distinguishing starch-fermenting gelatin-liquefying corynebacteria from *C. diphtheriae* var. *gravis* (Jebb, 1948).

Summary

A plate test for nitrate reduction is described. The test is based on the oxidation of haemoglobin to methaemoglobin by nitrites and is carried out on a blood agar medium containing potassium nitrate. Colonies of nitrate-reducing organisms are surrounded by an area of green discoloration.

The plate test is simple to carry out and has some advantages over the Griess-Ilosvay method of testing for nitrate reduction.

My thanks are due to Mr. H. A. Tarr for the photographs.

REFERENCES

Conn, H. J. (1936). *J. Bact.*, **31**, 225.
Jebb, W. H. H. (1948). *J. Path. Bact.*, **60**, 403.
Wilson, G. S., and Miles, A. A. (1946). Topley and Wilson's *Principles of Bacteriology and Immunity*, 3rd ed., vol. 1, p. 368. London.
ZoBell, C. E. (1932). *J. Bact.*, **24**, 273.

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THE DESTRUCTIVE EFFECT OF TRACES OF ZINC SALTS ON COMPLEMENT

BY

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From the Medical Research Council V.D. Reference Laboratory

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Price (1949) has advocated a method of complement titration in which graded complement dilutions are exposed to the action of (*a*) saline, (*b*) normal serum and antigen, and (*c*) normal serum under the conditions used in the actual test. During the complement titration for a gonococcal complement fixation test, the highest dilution of complement giving sparkling lysis in (*a*) was unusually low, whereas (*b*) and (*c*) gave average titres for the complement being used. This discrepancy was thought to merit investigation.

The complement was the pooled serum of about 70 large male guinea-pigs, preserved by Richardson's (1941) method. The haemolytic immune body was also part of a large pool. As both these reagents had been in general use in the laboratory and no previous difficulties had been encountered, it was thought they could be exonerated. The saline was freshly prepared from A.R. sodium chloride ; on testing for zinc with sodium di-ethyl-dithiocarbamate, as recommended by Mackie and McCartney (1945), a definite turbidity was produced.

Parallel complement titrations were then put up with the same complement, amboceptor, and sheep cells for each, but using saline prepared from the NaCl-containing zinc and from a batch from another maker which gave no turbidity with the zinc reagent. The total volume of the reagents was kept constant by varying the volume of saline in each mixture. The highest dilutions of complement giving complete lysis were:

Complement	Zinc-free Saline	Saline Containing Zn
(*a*) In the presence of saline alone	1/40	1/30
(*b*) In the presence of normal serum, antigen, and saline ..	1/35	1/35
(*c*) In the presence of normal serum and saline	1/45	1/45

Thus the same pattern of results was reproduced experimentally.

The following practical aspects of the destructive effect of zinc on complement, which might affect complement fixation tests, were investigated. The minimum inhibitory dose of zinc ; the effect of time and temperature of incubation ; the neutralization of the destructive effect by serum ; the effect of zinc-containing NaCl on the preservation of complement by Richardson's method ; the estimation of traces of zinc in sodium chloride.

Technique and Reagents

The same basic technique was used throughout these experiments. The unit volume used was 0.11 ml., being measured by a teated Pasteur pipette calibrated to deliver this volume. The reagents were made up to a total of three volumes with zinc-free saline, incubated in a 37° C. water bath for one hour unless otherwise stated, and one volume of a fresh 2½% sheep cell suspension, standardized by a haematocrit method (Price and Wilkinson, 1947) and sensitized with 5 M.H.D. rabbit anti-sheep cell haemolysin added to each tube. After 30 minutes' further incubation at 37° C., the tubes were centrifuged and the degree of lysis estimated by comparison with freshly prepared standards ranging from 0–100% lysis in increments of 10%. For these comparisons 75 × 5 mm. tubes of uniform bore were used. All experiments were put up in duplicate.

Complement.—This was preserved by Richardson's method, and each batch was used within a month of preparation.

Stock Zinc Solution.—This was prepared from A.R. zinc acetate and contained 1 mg. Zn/ml. The acetate was chosen, as Wright and MacCallum (1922) have shown that this ion was the least destructive of complement of any they studied.

Results

Minimum Inhibitory Amount of Zinc.—One volume of complement 1 in 80, 1 volume of saline containing 0.01–1.0 mg. Zn/100 ml., and 1 volume of zinc-free saline were incubated at 37° C. for an hour. One volume of sensitized cells was added and the degree of lysis estimated after a further 30 minutes at 37° C. The results are shown in Table I.

TABLE I

DETERMINATION OF THE MINIMUM INHIBITORY LEVEL OF ZINC

Zinc Content of Saline (mg./100 ml.)	Haemolysis (%)
1.0	0
0.5	22.5
0.25	67.5
0.10	80
0.075	85
0.05	87.5
0.025	92.5
0.01	95
Control (no zinc)	95

The figures (Table I) show that complement destruction first becomes appreciable with amounts of zinc between 0.05 and 0.1 mg./100 ml. corresponding to 0.0014–0.0028% Zn in NaCl. Mackie and McCartney (1945) quote unpublished work by Richardson, who found the maximum permissible amount of zinc in NaCl to be 0.0022%. Lührs (1939) showed that 1.0–2.0 ml. of a 1/10,000 solution of colloidal zinc oxide caused complete inhibition of lysis by 1 ml. of 2% guinea-pig serum.

The minimum inhibitory dose of zinc varies with the amount of complement in the reaction mixture. In the experiment described above, the dose of complement (1/80) was just insufficient to give complete lysis of the control. Using the same complement diluted 1 in 50, the minimum inhibitory dose of zinc was found to be between 1 volume of 0.25 mg. Zn/100 ml. and 0.5 mg. Zn/100 ml. saline.

Effect of Duration and Temperature of Incubation.—In a similar experiment 1 volume of complement diluted 1 in 80 was exposed to the action of 1 volume of saline containing 0.2 mg. Zn/100 ml. for periods of 0–120 minutes at 37° C. before sensitized cells were added. After one hour enough complement remained in the zinc-containing tubes to give 31% lysis, while the control (zinc-free) tubes showed 64% lysis. After two hours the respective figures were 2.5% and 31%.

The temperature of incubation was also of importance. Destruction was slight at 6° C. and 18° C., but considerable at 37° C.

Neutralization of Destructive Effect by Serum.—Two parallel sets of tubes were put up containing 1 volume of complement diluted 1 in 80 and 1 volume of pooled human serum inactivated at 56° C. for 30 minutes in dilutions from 1 in 10 to 1 in 300 in saline. To one set of tubes was added 1 volume of saline containing 0.5 mg. Zn/100 ml. and to the other set 1 volume of zinc-free saline. Both sets were incubated at 37° C. for one hour, and the amount of lysis estimated after a further 30 minutes at 37° C. after the addition of sensitized cells. These results are shown in Table II.

TABLE II

THE PROTECTIVE ACTION OF SERUM ON THE INACTIVATION OF COMPLEMENT BY ZINC

Dilution of Serum	Lysis (%)	
	Zinc	Control (No Zinc)
1 in 10	85	85
1 in 20	70	95
1 in 40	30	92.5
1 in 80	7	85
1 in 120	0	72.5
1 in 160	0	70
1 in 200	0	57.5
1 in 250	0	60
1 in 300	0	55
Control (no serum) 0%		

These figures show that the presence of serum in the reaction mixture affords considerable protection to complement in the presence of relatively large amounts of zinc, in this instance about six times the minimum inhibitory dose.

The means by which serum exerts this protective action is not clear, and it is hoped to investigate this question further. It raises one point of practical importance. If a complement titration is carried out using saline contaminated with zinc salts and the dose of complement is estimated from the highest dilution of complement giving complete lysis in the presence of saline alone, the value arrived at will be too large, since in the test proper the tubes will contain sufficient serum to exert a protective effect. This excess of complement will lower the sensitivity of the test. If complement doses are gauged from a consideration of complement dilutions exposed under the conditions of the test to the presence of (*a*) normal serum, and (*b*) normal serum plus antigen, as advocated by Price (1949), this underestimate of complement activity is avoided.

Effect of Zinc-containing NaCl on Preservation.—The reagents used in the Richardson method for preserving complement are dissolved in saturated aqueous NaCl. If this contained zinc, the concentrated solution might be expected to introduce relatively large amounts of zinc into the complement, although the serum proteins might offset the destructive effect. Portions of the same pool of guinea-pig serum were preserved with

reagents dissolved in (*a*) zinc-free saline, and (*b*) saline from NaCl containing 0.004% Zn. The lytic effect of dilutions of both specimens of complement against the same batch of sensitized cells was determined after incubation at 37° C. for one hour before the addition of cells. The amount of lysis produced by the complement preserved with the zinc-containing salt was slightly less than the control, but the difference was not considered significant.

The Estimation of Traces of Zinc in NaCl.—The method described by Atkins (1935), which will detect as little as 0.06 parts per million of zinc in water, was used. A series of standard solutions containing from 1.0 to 0.01 mg. Zn in 50 ml. volumes of distilled water was prepared in 6 in. × 1 in. tubes of uniform bore. A blank containing 50 ml. of the distilled water used for preparing the solutions was included. The unknown consists of 50 ml. of a 1% solution of the NaCl under test. To each tube was added 5.0 ml. of a freshly prepared 0.1% aqueous solution of sodium di-ethyl-dithiocarbamate (B.D.H. reagent for delicate analysis). The contents of the tubes were mixed, allowed to stand for 30 minutes, and any turbidity produced compared with the standards. The turbidity produced by zinc is clarified by the addition of 0.880 NH_4OH, whereas that produced by copper is not. Copper and iron salts produce a brownish opalescence which may interfere with the estimation.

Eight batches of NaCl of A.R. purity were examined. In six of these no zinc could be detected. In one, a trace was present, well below 0.002%, while the remaining specimen was found to contain 0.004% Zn.

Summary

The presence of zinc in NaCl in amounts greater than 0.002% has been found to reduce the haemolytic activity of complement.

The effect of duration and temperature of incubation on this action of zinc has been examined.

The presence of serum has been found to lessen the destructive effect of zinc.

A simple method of estimating traces of zinc in NaCl is suggested.

My thanks are due to Dr. I. N. Orpwood Price, Director of the Medical Research Council V.D. Reference Laboratory, for his constant encouragement and advice during the course of this investigation, part of which was carried out during the tenure of an Insole Scholarship awarded by the British Medical Association.

References

Atkins, W. R. G. (1935). *Analyst*, **60**, 400.
Lührs, E. (1939). *Z. ImmunForsch.*, **95**, 70.
Mackie, T. J., and McCartney, J. E. (1945). *Handbook of Practical Bacteriology*, 7th ed., p. 671. Edinburgh.
Price, I. N. O. (1949). *Brit. J. vener. Dis.*, **25**, 157.
—— and Wilkinson, A. E. (1947). Ibid., **23**, 124.
Richardson, G. M. (1941). *Lancet*, **2**, 696.
Wright, H. D., and MacCallum, P. (1922). *J. Path. Bact.*, **25**, 316.

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TECHNICAL METHODS

A MAGNETICALLY OPERATED BLOOD CELL PIPETTE SHAKER

BY

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A blood cell pipette shaking machine has been reported by Parry and Simmonds (1950). The approximate cost of a commercial model was estimated at £70. Small laboratories, however, find this sum considerable in a limited budget. Using a different principle, a small instrument has been designed and tested here which shakes pipettes in a vertical plane and at the same time causes them to rotate. Early defects have now been eliminated, and it is considered that this instrument used in the way described will give highly satisfactory results. The great advantage is the low cost of the instrument; a provisional estimate is £7 or less.

Description of the Instrument

The instrument embodies two holders each capable of carrying four pipettes which are shaken by means of a special type A.C. magnet. This magnet is in effect a modified mains transformer built from U and T shaped laminations. When a normal transformer is assembled the laminations are interlaced by alternating the positions of the U and T shapes with respect to the coil. In this application the U laminations are first modified by removing an eighth of an inch from each leg of the U, and then assembled into a stack. The T laminations (unmodified) are assembled together in one stack and inserted into the former on which the magnet or "transformer" coil has been wound. The U laminations are arranged to encircle the coil in the normal manner. This assembly is fastened together by standard transformer clamping frames, giving what appears to be a transformer with eighth-of-an-inch air gaps in the iron circuit. The magnet coil comprises a single winding of 24 S.W.G. enamelled wire, the former being fully wound as no secondary winding is required.

On the top of the magnet is pivoted a length of ½ in. by 3/16 in. dural bar which has mounted at one end a piece of mild steel sheet 1/16 in. thick. This piece of steel is arranged so that it bridges the top one of the two gaps in the iron circuit, and it acts as the armature of the magnet. The other gap in the laminations ensures that the flux is distributed evenly round the whole of the iron circuit.

A small block of rubber is inserted between the dural bar and the magnet laminations. This piece of rubber is kept under slight compression by a strip of brass mounted over the armature which restricts the movement of the dural bar. The rubber, being under compression, acts as the return spring for the bar when it has been attracted

to the magnet core. A piece of thin rubber attached to the underside of the armature behaves as a residual stud, thereby preventing the armature from making metallic contact with the magnet core. This ensures that the armature is released rapidly as the current falls to zero, and further that the shaker is quiet in operation.

Attached to the other end of the dural bar there is a length of beryllium copper strip in the shape of a U. This U piece carries two holders each capable of taking up to

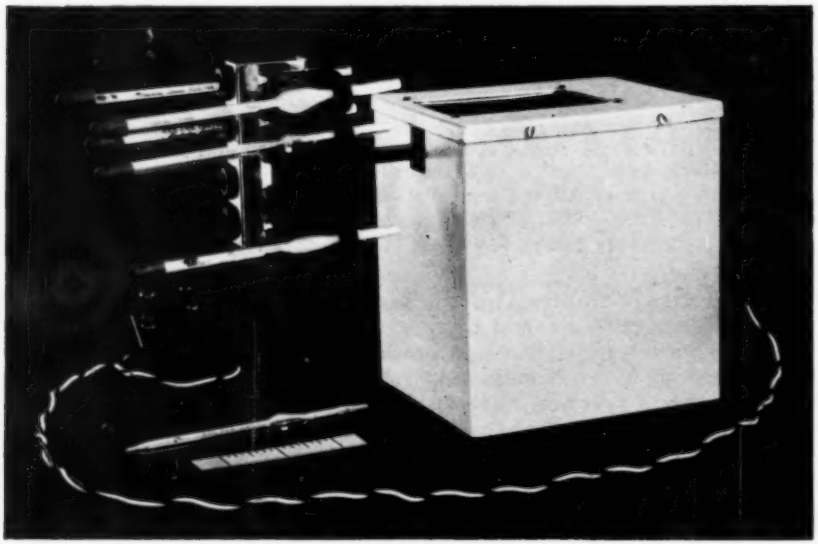

FIG. 1*a*.

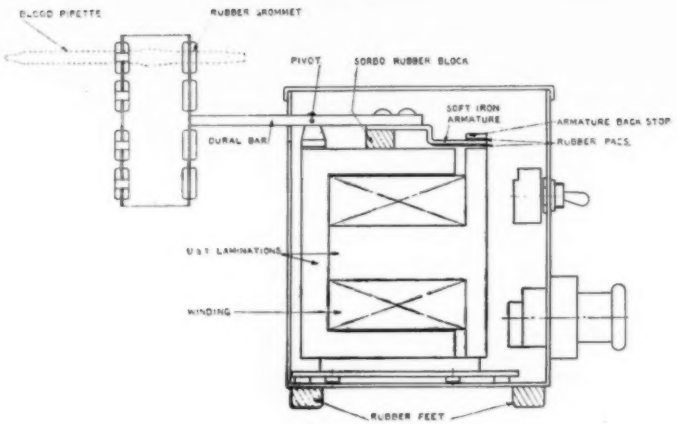

FIG. 1*b*.

FIGS. 1*a* and 1*b*.—The shaker and a diagram showing its construction.

four pipettes. A pipette is supported on either side of the bulb by a rubber grommet, one grommet being slit to allow the insertion of the pipette into the holder. This type of support will take pipettes of varying length.

When the magnet coil is energized by the 230 volts 50 cycle mains, the flux across the air gap rises and decays one hundred times per second. The armature follows the growth and decay of this flux and consequently the dural bar vibrates in the vertical plane one hundred times per second. This vibration is transmitted to the holders via the beryllium copper spring, and further, due to the resilience of the spring, a small horizontal vibration is imparted to the holders.

The pipettes are loosely held in their holders. The resultant force from the two vibrations causes the pipettes to roll round the inside of the rubber grommets. This rotary motion is very pronounced, and in fact the pipettes rotate rapidly while they are being shaken vigorously in the vertical plane.

Method of Use

In this instrument the ends of the pipettes are free and are not held in concave rubber seatings to prevent fluid loss as in the machine previously described. One of the difficulties encountered was the considerable fluid loss from the ends of the pipette. This is overcome by the use of a " rubber policeman " (Fig. 1) placed over the tip. In practice it has been found that it is only necessary to close the tip of the pipette ; the other end is left free. Small perforations are punched through the rubber near the tip. This is necessary to allow the displaced air in the " rubber policeman " to escape when it is slipped over the tip of the pipette, otherwise the fluid contents of the capillary portion of the pipette are displaced into the bulb and alter the dilution. It has been found that with practice in slipping on the rubber there is no fluid displacement, and when the rubber is in place there is no fluid loss however long the pipette is shaken. By placing the perforated " rubber policeman " on the dilution pipettes at the time of taking the blood sample, accidental loss of the contents is avoided during transit from the ward or clinic to the laboratory.

The pipettes are placed in the shaker by inserting the blunt end of the pipette through the circular rubber grommet and pressing the capillary portion of the pipette into the slit rubber grommet. The instrument is switched on. Pipettes can, however, be removed and inserted while the shaker is in motion. The shaker has been in use for two months, and, although no statistical work has been undertaken in connexion with this instrument, it is thought that satisfactory mixing is attained in two minutes.

Summary

A magnetically operated blood cell pipette shaker is described. The shaker holds up to eight pipettes at one time in two rows of four. The pipettes are shaken vigorously in a vertical plane and at the same time rotate rapidly.

The use of perforated " rubber policemen " in order to prevent fluid loss from the pipettes is described.

Acknowledgment is made to the Director, Sir John Cockcroft, Atomic Energy Research Establishment, Harwell, for his permission to publish this article; also to Dr. K. Williams, Principal Medical Officer, for assistance in the preparation of this paper.

Reference

Parry, K. C., and Simmonds, G. E. (1950). *J. clin. Path.*, 3, 164.

J. clin. Path. (1950), 3, 370.

BLOOD PIPETTE SHAKING MACHINE MARK II

BY

K. C. PARRY

From the Medical Division, Atomic Energy Research Establishment, Harwell

(RECEIVED FOR PUBLICATION JUNE 22, 1950)

The blood pipette shaking machine previously reported (Parry and Simmonds, 1950) has been modified to incorporate various improvements. In the new machine (1) the pipettes lie in the same plane and there is greater ease of insertion. (2) The speed of rotary movement has been increased and that of tilting decreased to reproduce

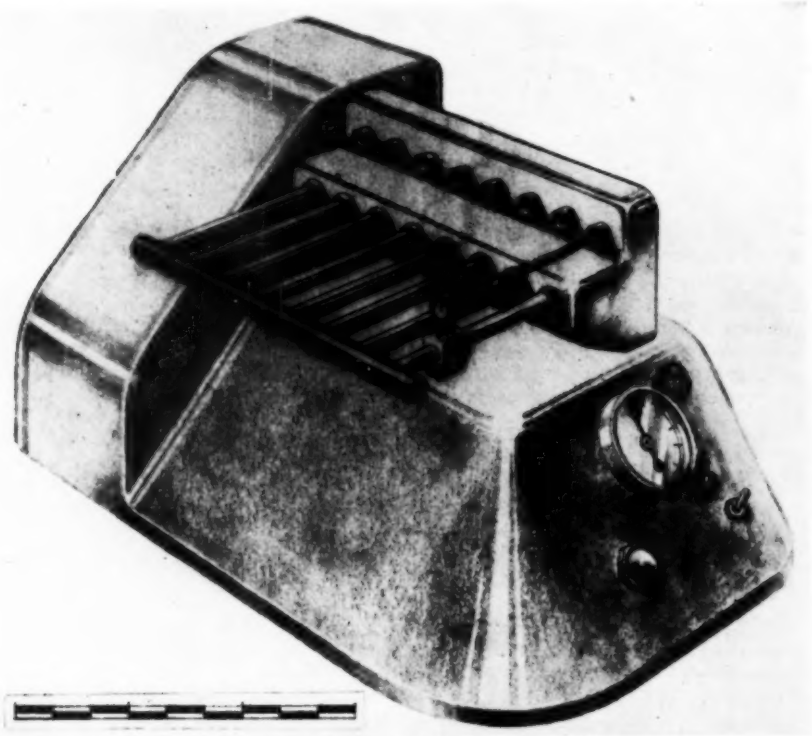

FIG. 1.—Blood pipette shaking machine mark II.

more exactly the motion imparted to pipettes in the hand shaking method. The speed of tilt is 30 per minute ; the pipettes reciprocate three times to each direction of tilt, so that the number of reciprocal revolutions is now 180 per minute. (3) Eight pipettes can be shaken at the same time. (4) Each pipette holder is numbered to assist in the identification of pipettes. (5) A light indicates when the machine is in circuit. (6) The cost is estimated at less than that of the previous model.

Acknowledgment is made to the Director, Sir John Cockcroft, Atomic Energy Research Establishment, Harwell, for his permission to publish this account.

REFERENCE

Parry, K. C., and Simmonds, G. E. (1950). *J. clin. Path.*, 3, 164.

MAR 15 1950
OCT 5 1950

J. clin. Path. (1950), 3, 372.

A SIMPLE INOCULATION RACK AND MODIFIED TECHNIQUE FOR RAPID SUBCULTURING AND ANTIBIOTIC TESTING

BY

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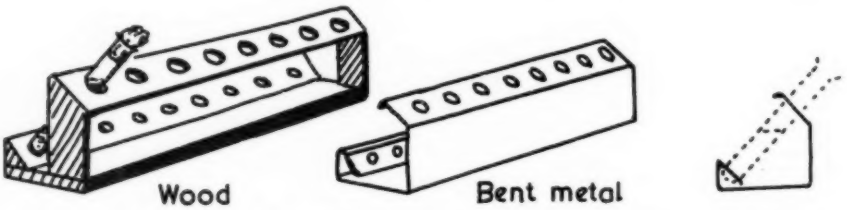
From the Serum Research Institute (Medical Research Council), Carshalton, Surrey

(RECEIVED FOR PUBLICATION JULY, 1950)

The test-tube rack usually used by bacteriologists to hold culture tubes was inherited from chemical laboratories and has disadvantages for bacteriological work in which the culture tube is held in a sloping position for inoculation. The inoculation rack described here has been in use for some years and has been found to be most efficient and to lend itself to a modified rapid technique for subculturing and for titrating antibiotics.

The tubes are held in a sloping position at an angle of about 45° to the vertical as shown in the figure. The advantage is that cotton wool plugs (or metal caps)



can be removed and pipetting and inoculating carried out while the tubes are still in the rack instead of being held in the hand. The slope prevents particles of contaminated dust falling into the culture medium as they are trapped in the top of the tube, and during incubation particles from the plug do not fall into the medium. The angle of slope of the tubes is the one normally used for inoculation or pipetting, so that if many tubes are to be inoculated or to receive serial dilutions of antibiotic, all the cotton wool plugs are removed and placed on a flamed surface, e.g., the bottom of an upturned wire mesh basket. Pipetting and inoculation can then be carried out at leisure without the risk of contamination from falling dust particles, and it is found in practice that, even when the experiment is interrupted and the tubes are left open for a time before the plugs are replaced, contamination does not occur. Flaming of the tops of tubes before and after inoculation is effective in destroying any contaminating particles.

A further advantage of the sloping racks is in the ease of examining the cultures after incubation. The rack need not be held up for inspection, but the slope of the

tubes is convenient for direct observation on the bench. For this purpose racks are painted a matt black so that turbidity is easily seen against the dark background of the base of the rack. For biochemical work white-painted racks make colour changes easy to detect without the necessity of holding racks up to the light.

The wide base of the rack makes it practically impossible to overturn the rack either on the bench or incubator no matter how carelessly it is handled. The construction of the rack is simple and inexpensive and may be of wood, or it may be made from a single piece of bent aluminium alloy or stainless steel.

Summary

A simple rack is described in which culture tubes are held in a sloping position suitable for inoculating and pipetting operations without the risk of air contamination or the need to hold tubes in the fingers. Besides the greater rapidity achieved in carrying out multiple subcultures and antibiotic tests and the decrease in the contamination of cultures, the inspection of cultures is accelerated and simplified.

MAR 15 1952

J. clin. Path. (1950), 3, 374.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 44th SCIENTIFIC MEETING

The forty-fourth scientific meeting was held at Leeds on July 20, 21, and 22 under the Presidency of Dr. Norah Schuster.

The Presidential Address

Dr. SCHUSTER's presidential address was entitled "Nec silet mors," and it told of the increasing demands which advances in medicine and surgery made upon pathologists who undertook necropsies; to-day they must needs pay attention to parts of anatomy which 25 years ago they could safely ignore, but as a profession doctors were always willing to learn.

A full medical and occupational history was essential for the pathologist at the time of the necropsy, otherwise there might be great wastage of material.

The presidential address will be reported in full in the year book of the Association of Clinical Pathologists. The year book will be published early in 1951.

The inaugural session was devoted to a consideration of allergic diseases of blood vessels.

Polyarteritis Nodosa

Dr. G. DISCOMBE (London) reviewed 16 proved and six suspected cases of polyarteritis nodosa. Laboratory investigations did not always help in the diagnosis, for biopsy was sometimes negative. Lesions might heal and, if in the kidney, could produce an essential hypertension, the changes of which might almost completely obliterate those of polyarteritis nodosa. Eosinophilia, although common and usually demonstrable by direct counts during the active phase, was not an essential part of the syndrome. Three cases were described to illustrate the variable symptomatology.

Dr. W. PAGEL (London) discussed some unusual aspects of the histology and the question of aetiology. Subendothelial swelling due to permeation of fluid seemed to be followed by exudation of fibrinogen with subsequent necrosis, mostly eccentric, of the vessel wall, a breach in which became plugged with fibrin. Fibrin was deposited, too, both inside and outside the necrotic wall. He preferred to reserve the term "fibrinoid" for changes which failed to show the staining affinities of fibrin, and which sometimes occurred in positions which did not always favour the assumption that one was dealing with fibrin which had lost its staining affinities. In his opinion the differences between polyarteritis nodosa and the "pararheumatic" diseases, including lupus erythematosus disseminatus, dermatomyositis, and temporal arteritis, concerned the natural history of the disease and the preferential site and distribution of the basic change rather than the basic histology, which appeared to be identical. In polyarteritis nodosa extramural foci involved the collagen fibres and were indistinguishable from the acute infiltrative changes in rheumatic fever, lupus erythematosus disseminatus, and dermatomyositis. Preservation of the argentaffin reticulum-fibrils with loss of staining affinities of the collagen fibres could be found in the lesions of polyarteritis nodosa, while giant cell granulomas, reminiscent of

Aschoff nodules and the picture of temporal arteritis, were not uncommonly found. In some instances healed polyarteritis nodosa appeared to be the basis of an eosinophil-celled alveolitis (Loeffler's syndrome) associated with exfoliative dermatitis and asthma (in one case terminated by cerebral haemorrhage). While in these cases the arterial changes appeared to be burnt out, the eosinophilia was active, so that it was not necessarily to be interpreted as a cellular reaction to local arterial necrosis. Intradermal injections of heat-killed streptococci in healthy persons and in rheumatic and other patients who had volunteered had established the significance of bacterial antigens in the reproduction of the changes characteristic of polyarteritis nodosa. Both the histological and experimental evidence supported the view that polyarteritis nodosa and the related diseases were an expression of the hypergic response to any aetiological factor, including bacterial (notably streptococcal) antigen.

Dr. I. RANNIE (Newcastle-upon-Tyne) described the value of biopsy. A positive result was obtained in eight out of twelve cases, the four unsuccessful biopsies being explained either by the unsuitability or insufficiency of the material submitted. In five of the eight positive instances biopsy was carried out for confirmation of diagnosis; four were taken from the muscle and one from a skin nodule. The diagnosis of polyarteritis nodosa was established as a chance biopsy finding in the remaining three cases, the organs submitted being respectively appendix, liver, and cervix uteri and ovaries. Muscle was the material of choice; a tender area should be chosen for excision, and a negative report should not be issued until serial sections had been studied.

Professor T. CRAWFORD (London), discussing the experimental production of polyarteritis nodosa, said that there were two main types which had led to the production of lesions in animals closely resembling those of polyarteritis in man. The first, introduced by German pathologists about 1930 and used extensively by Rich and Gregory in the U.S.A., consisted of the administration of foreign protein to a suitable animal such as the rabbit. The second, described in this country by Wilson and Byron, depended upon the production of hypertension by some procedure such as the clipping of a renal artery in rats. The interpretation of these experiments which the speaker favoured was that the first procedure led to a tissue-antigen-antibody reaction characterized by necrosis and fibrin deposition in the vessel walls and a cellular reaction around the injured area, whereas with the second procedure sudden elevation of blood pressure caused damage to the less well supported arteries and this again led to necrosis and fibrin deposition in the vessel wall, with a secondary cellular response as in the former lesion. When considered in conjunction with knowledge gained from clinical sources, the former of these experimental procedures seemed to constitute a closer parallel to the conditions of the human disease than did the latter. Study of these lesions might well lead to a better understanding of the human disease and of the effects of various agents upon it.

The sessions which followed provided a varied programme.

The Pathology of Measles

Dr. G. B. S. ROBERTS (Glasgow) reported the necropsy findings in a child who died during the prodromal stage of measles with Koplik's spots. Convalescent measles serum had been given 12 days previously. Death from a fulminating pneumococcal meningitis occurred before the appearance of the rash. The lymphoid tissue throughout the body showed hyperplasia of the germinal centres with the formation of large multinucleate giant cells of the Warthin-Finkeldy type. A second case was mentioned, in which a cervical lymph node, removed at operation, was found to show typical Warthin-Finkeldy giant cells. Although this child had been

exposed, a clinical attack of measles did not develop, so that it appeared possible that infection could produce histological changes without any clinical manifestation of the disease. Giant cells found in the lung of the fatal case contained large numbers of acidophilic intracytoplasmic inclusion bodies which showed great variation in size and shape.

An attempt to communicate the disease to rabbits with intravenous inoculation of citrated blood taken from patients on the day of appearance of the rash was unsuccessful.

The Study of Malignant Cells in the Nose and Throat

Dr. I. FRIEDMANN (London) gave an account of his experiences with the Papanicolaou method of diagnosis as applied to laryngeal and nasal secretions collected on gauze or cotton-wool swabs attached to long straight or curved swab-holders or wooden applicators. The Papanicolaou smear was at its best in squamous-cell carcinoma, in particular of the larynx, whether intrinsic or extrinsic. In inflammatory conditions of the sinuses hyperplastic epithelial cells and histiocytes, often multinucleated, caused difficulty.

In spite of its limitations the method as applied to the diagnosis of tumours of the nose and throat was of real value, and it had proved useful as a presumptive test of malignancy in ulcerative conditions of doubtful aetiology.

Experimental Studies on Prothrombin

Dr. T. B. MAGATH (Rochester, Minnesota), who conveyed to the Association greetings from the American Society of Clinical Pathologists, discussed various phases of the prothrombin problem, and described a convenient one-stage technique.

Plasma, serum, and platelet extracts possessed activity concerned with the conversion of prothrombin to thrombin. Since zymin plasma produced a conversion deficiency which differed from that produced by ageing or by ammonia, it was apparent that there was more than one conversion factor. An apparently low prothrombin value might be caused by a lack of one or more conversion factors. The thromboplastic effect of platelets was due in part to an associated prothrombin conversion factor, while complement had been shown to possess properties very like some conversion factors in plasma. Several experiments were described and critical values for dicoumarol therapy given.

"Sixth Day Disease"

Drs. R. AIDIN and G. H. TOVEY (Bristol) gave an account of "sixth day disease," a syndrome consisting of prematurity (which occurred in all but one instance), jaundice, and kernicterus, with death usually about the sixth day of life. In their experience, kernicterus was most commonly found in this condition, 25 examples, unassociated with haemolytic disease, having been found in a series of necropsies on 144 premature infants. While the naked eye appearance was indistinguishable from the kernicterus found in haemolytic disease, clinical, necropsy, histological, and serological studies failed to support a diagnosis of haemolytic disease; there was no evidence of sepsis or syphilis.

Clearance of *Staph. pyogenes* from the Nose

Dr. R. W. FAIRBROTHER (Manchester) dealt with the problem of the clearance of *Staphylococcus pyogenes* from the nose. The application of penicillin cream had proved of value, but the clearance was usually only temporary, as *Staphylococcus pyogenes* tended to recur a few weeks after the cessation of treatment, in spite of

repeated courses of the cream. In some instances the fresh organisms were of a type different from that of the original staphylococci. Some insensitive strains were encountered.

Amoebiasis in Britain

Air Vice-Marshal T. C. St. C. Morton (Wendover) discussed the carrier rate of amoebiasis especially in Britain. It varied considerably, not only from country to country but from town to town, and was dependent more on the state of hygiene of the community than on the geographical site. It had to be clearly separated from the incidence of actual amoebic dysentery, which in the United Kingdom was extremely low. The reason for this anomaly was probably due to the fact that in the Tropics the opportunities for the free interchange of intestinal flora were considerably greater than in the United Kingdom.

Sixteen out of 1,000 R.A.F. apprentices were found to be carrying *E. histolytica* cysts. Three cases of amoebic dysentery, one fatal, were encountered in R.A.F. personnel who had never left Britain, but it was significant that two of them had had contact with patients who were suffering from dysentery contracted overseas.

Dr. T. B. Magath believed that the incidence of carriers in the United States was of a similar order—namely, about 1.6 to 2.0%, although textbooks often quoted a carrier rate of 10%. It took many years of experience to become proficient in recognizing pathogenic amoebae, and he believed that iron haematoxylin was the best stain for identifying them. Epidemics, in his experience, were almost always waterborne.

The Distribution and Frequency of Non-pulmonary Tuberculosis

Dr. G. S. Wilson (London) gave an account of the survey that was carried out in England and Wales during 1943–5 into the distribution and frequency of non-pulmonary tuberculosis caused by the bovine type of bacillus. The survey was an excellent example of what could be achieved by co-operation between different laboratories making use of ordinary routine material, and Dr. Wilson took the opportunity of thanking the 149 pathologists who had gone to the trouble of collecting specimens for examination by the bacteriologists. In England strains from 994 patients had been examined; of these, 26.3% belonged to the bovine type. The percentage distribution according to site of disease was: meninges 27.1; cervical glands 46.5; bones and joints 10.8; genito-urinary system 12.8; miscellaneous 10.6. The proportion of bovine-type strains was highest in the second quinquennium of life, and higher among females than males. It was calculated that in England and Wales during 1944 about 24% of all cases of non-pulmonary tuberculosis were of bovine origin and that 1,300 to 1,400 persons died of milk-borne tuberculosis. In conclusion Dr. Wilson drew attention to the high proportion of mistaken diagnoses in cervical adenitis and suggested that further work was required to distinguish the tuberculous from other forms of the disease.

Dr. G. Stewart Smith (Exeter) spoke on the problem of non-pulmonary tuberculosis as seen in an urban and rural community in the south-west.

The Epidemiology of the Coxsackie Virus

Dr. A. J. Rhodes (Toronto) gave an account of the epidemiology of the Coxsackie virus and of a recent epidemic in Dufferin county, in which both the viruses of poliomyelitis and of the Coxsackie infection appeared to take part; it appeared that the two viruses possessed a similar epidemiology. Difficulties in diagnosis of virus infections were dealt with and many valuable practical hints given.

Dr. F. O. MacCallum (Colindale)

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described briefly the results of recent investigations made on material sent in to the Virus Reference Laboratory, and the problems of diagnosis to-day.

The Interpretation of Blood Cholesterol Estimations

Dr. J. B. FOOTE (London) discussed the interpretation of blood cholesterol estimations, favouring the method of Bloor because of its simplicity, reproducibility, and speed. However, in a series of 33 male and 17 female medical students, he and Dr. Merivale had found the normal range to be 150 to 350 mg.%. The method had the disadvantage of not separating free cholesterol from its esters, and rate of colour production and intensity of colour were not the same for free and ester cholesterol. Nevertheless, by careful attention to technique, the errors tended to cancel out. Despite the wide normal range they found that the estimation was of limited value in the diagnosis and control of treatment in 22 cases of myxoedema, but that in 49 examples of thyrotoxicosis the overlap with the normal was so great (68.8%) that the use of serum cholesterol estimations in its diagnosis was precluded. Thirty cases of hepatitis investigated at the Westminster Hospital showed a wide scatter and 90% within the normal range.

Polarographic Serum Waves in Prostatic Disease

Dr. P. G. WALKER and Mr. R. D. WILKINS (London) compared the polarographic serum waves with the total and formol-stable serum acid phosphatases in a series of cases diagnosed clinically as malignant disease of the prostate: 55% had abnormal polarographic waves and 52% abnormal phosphatase values. However, these did not necessarily coincide, for, while in 25% both values were high and in 25% both values were within the normal range, in the remaining cases one or other determination was above the normal.

Acute Porphyria

Professor C. H. GRAY (London) made observations on acute porphyria, eight examples of which had illustrated the great variation in the clinical picture and had supported the view that this condition was wholly different from congenital, light-sensitive porphyria. In all probability congenital porphyria is a genetically determined enzyme abnormality, probably localized in the bone marrow leading to production of Type 1 porphyrins in excess during erythropoiesis. In acute porphyria there might be an excessive production of mono-pyrrolic porphyrin precursors, possibly in sites of porphyrin synthesis other than the bone marrow. The clinical condition might be brought about by direct action of the porphyrins on the central nervous system, by primary changes in the central nervous system resulting in the excessive production of porphyrin precursors, or by the direct action of the porphyrin precursors themselves on the central nervous system.

Oliguria and Anuria

Drs. E. M. DARMADY, J. HARKNESS, and A. RICHARDSON JONES (Portsmouth) drew attention to the fact that oliguria and anuria might follow a large number of precipitating causes, over 60 of which had already been described. The course of the majority of cases could be divided into three phases. The phase of onset, frequently taking the form of shock, showed oliguria and haemoconcentration. This was followed by the anuric or oliguric phase, in which there was a rapid rise in blood urea, a fall in serum sodium and chlorides, and a rise in serum potassium, with its danger of acute

cardiac failure. It was important to restrict fluids in this second stage. The two methods of controlling accumulation of metabolites, either by dialysis, as a temporary measure, or by a high fat and carbohydrate diet, were reviewed.

In the third, or diuretic, phase, the authors drew attention to the rise in urinary output, which might be extremely sudden in onset and often accompanied by a serious loss of potassium. They had had difficulty at times in keeping pace with the diuresis and with the potassium deficiency. Faulty hydration was the important aspect of the disease.

Agranulocytosis of Glandular Fever

Dr. C. J. YOUNG (Bradford), in considering the agranulocytosis of glandular fever, expressed the opinion that this was a diagnosis which was commonly missed. When looked for, three examples were seen in a period of just over two years, one of which yielded new information on the nature of the condition. In this case the marrow was found to be normal, but the granulopenia persisted in the blood and was still present two and a half years later. The condition was liable to be confused with drug agranulocytosis, but there were four features which helped to distinguish it, although none were specific; these were the Paul–Bunnell test, the constant presence of a few polymorphs in the blood, the normal bone marrow, and the absence of fatalities.

Dr. Young suggested that the mode of production of the agranulocytosis was different from that caused by drugs, being due to the unduly rapid removal of polymorphs from the blood, and not to a failure to produce them in the marrow. It was recommended that glandular fever should be looked for in agranulocytosis which might be due to other causes. When due to this condition the prognosis was good, and the patient need not be deprived of a suspected drug, if this would be to his disadvantage. Bacterial invasion could, however, occur.

The Relationship between Carcinoma *in Situ* of the Cervix and Invasive Carcinoma

Dr. OSCAR B. HUNTER, jun. (Washington, D.C.) referred to reports that carcinoma *in situ* in the cervix developed into invasive carcinoma. However, proof that carcinoma *in situ* was actually the precursor of invasive carcinoma was presumptive, because of the nature of the lesion. Biopsies might miss an invasive carcinoma, whereas in attempting to examine the cervix adequately conization sections might completely destroy the lesion. Other evidence of the relationships existed in that the statistical incidence of cases of the two lesions were approximately the same. Age incidence statistics, however, revealed that carcinoma *in situ* averaged round 35 years of age, whereas invasive carcinoma was 45 years. From experience of 20 cases of clinically occult cervical lesions discovered by positive cytological examination, he had discovered extremely early evidence of invasion in what was otherwise carcinoma *in situ*. This evidence had been found after serial sectioning of the cervices. More clear-cut proof that carcinoma *in situ* ultimately developed into invasive carcinoma could probably be made by further study of very early cases of carcinoma *in situ*. The sheer weight of evidence might then force the proper conclusion.

Laboratory Investigation of Haemophilia

Dr. C. MERSKEY (Oxford) described laboratory findings in cases of haemophilia.

This paper is published in full on page 301.

In Vitro Tests for Haemolysins in Cases of Cold Haemoglobinuria

Dr. J. V. DACIE (London) referred to a number of discrepancies concerning the behaviour *in vitro* of cold haemolysins in cases of cold haemoglobinuria, as revealed in a survey of the literature. Particularly was this true of the effects of carbon dioxide, the role of complement in the cold sensitizing phase, the thermolability of the haemolysins, and the optimum time for sensitization. There were also suggestions that the patient's serum might sometimes be deficient in complement. He had studied the sera of three patients; in two of them cold haemolysins were associated with cold haemagglutinins at high titres. The *p*H ranges for the three sera differed; in one case haemolysis did not take place unless the serum was acidified to within the *p*H range 7.5–6.5. It could be demonstrated that *p*H affected the adsorption of the haemolysins in the cold sensitizing phase. In two out of the three sera the presence of fresh serum in the cold phase was found to be necessary for the adsorption of the haemolysin. The serum of one patient was deficient in complement.

Dr. Dacie concluded that as real differences existed between the behaviour *in vitro* of cold haemolysins no test should be regarded as negative unless it had been carried out with serum acidified within the *p*H range 6.5–7.5 with a further tube set up in which an equal volume of fresh normal serum had been added to the patient's serum, so as to provide an adequate concentration of complement. The patient's serum should never be heated, and fresh serum must be present in both the cold and the warm phases.

Speakers at the dinner of the Association, which was held on July 21, included Professor Matthew Stewart and Dr. Robert A. Moore, Professor of Pathology at the Washington University of St. Louis, Missouri, and Secretary of the American Board of Pathology.

J. clin. Path. (1950), **3**, 381.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Clinical Observations on Infectious Mononucleosis in Adults. MÜLLER, G. (1950). *Z. klin. Med.*, **146**, 1.

From the Medical Clinic, Bremen, the author discusses at some length all that is at present known about glandular fever (Pfeiffer's disease). After an exhaustive historical résumé extending from the initial description given by Pfeiffer in 1889 to the present time, with particular reference to the angiose form (which appears to have become much more frequent of late), he proceeds to a minute description of the clinical and pathological findings in 37 cases observed by him in 1948. It is pointed out that the faucial angina, which may be difficult to distinguish from that of diphtheria, is due to hyperplasia of the lymphoid tissue of the pharynx, and that there is no one type of lesion which can be regarded as pathognomonic of glandular fever.

Points of interest from this long paper which may, perhaps, not be generally familiar even to experts in this field may be summarized as follows: (1) Initial symptoms may occur for at least a week before the characteristic enlargement of lymph nodes takes place. (2) Splenic enlargement is likely to be gross in about half the cases and some degree will be present in at least two-thirds. (3) Changes in the leucocyte count are not accompanied by an anaemia, any alteration in the erythrocytes being quite rare in glandular fever. (4) The Paul–Bunnell reaction may be positive as early as the fourth day of the disease and is likely to become negative after a month. [This finding is not the rule in the type of case lately prevalent in London.] (5) The sternal marrow shows a shift of myeloid cells towards the immature side, but there is no noticeable increase in plasma cells. From this it is deduced that changes observed in the peripheral blood are due to metaplasia originating in lymphoid tissue and not to an alteration in the haematopoietic powers of the marrow; glandular fever is not a blood disease. (6) The administration of sulphonamides, etc., appears to be without benefit. (7) Much difficulty may be encountered in distinguishing the angina of glandular fever from that of true diphtheria especially when, as not infrequently happens, diphtheria bacilli are present in the fauces. [Many cases of glandular fever are in fact sent into fever hospitals as diphtheria.] As distinguishing features, the author mentions absence of the typical diphtheritic foetor and the characteristic lumpy lymph-node enlargement as contrasted with the toxic oedema (bull-neck) of diphtheria. (8) Return to normality is stated to be rapid [an observation not confirmed in Great Britain] and the prognosis uniformly good.

[Only a selection of points has been given from an elaborate clinical paper which seems to give the most complete account of glandular fever at present to hand, though it contains nothing which could rank as a new discovery.]

Jos. B. Ellison.

A New Sulfonamide (Gantrisin). Studies on Solubility, Absorption and Excretion. SVEC, F. A., RHOADS, P. S., and ROHR, J. H. (1950). *Arch. intern. Med.*, **85**, 83.

A report is presented on the solubility, absorption, and excretion of a new sulphonamide, 3 : 4 - dimethyl - 5 - sulphan lamido-*iso*xazole, formerly known as "Nu 445" and now called "gantrisin." The authors have used this sulphonamide in the treatment of 300 patients and have not found any evidence of renal damage due to the drug. It is more soluble than sulphadiazine in urine at about *p*H 6.3, having a solubility of 327 mg. per 100 ml. compared with 13 mg. per 100 ml. for sulphadiazine. The solubility of sulphadiazine only approaches that of gantrisin when the *p*H is raised to 7 or 8. The free form of gantrisin is more soluble than the acetylated form up to *p*H 6.6, whereas the opposite is true of sulphadiazine.

Gantrisin was given orally, intramuscularly, and intravenously, the dosage varying from 1 to 2 g. every 4 hours. A maximum total concentration in the blood of 9 to 17 mg. per 100 ml. was obtained, according to the route of administration. The acetylated portion varied between 28% and 35% of the total. Excretion in the urine followed the usual pattern of sulphonamide excretion, the major portion of the dose, 80 to 90%, being excreted within 48 hours. The compound was administered parenterally as a 40% solution of the ethanolamine or diethanolamine salts, which produced no local reactions.

R. Wien.

Chloromycetin (Chloramphenicol) in the Treatment of Infections. HEWITT, W. L., and WILLIAMS, B. (1950). *New Engl. J. Med.*, **242**, 119.

Tests for bacterial sensitivity to chloramphenicol ("chloromycetin") were performed *in vitro*, and from 2 to 39 strains of each organism were employed. The majority of the strains of *Pseudomonas aeruginosa* and about half the strains of proteus proved to be resistant, whereas the majority of the strains of streptococcus, pneumococcus, *Klebsiella pneumoniae*, *Aerobacter aerogenes*, *Bacterium coli*, enterococcus, and *Staphylococcus aureus* were sensitive to less than 5 μg. per ml. Therefore chloramphenicol has a wide range of *in vitro* bacteriostatic activity against pathogenic Gram-positive cocci and Gram-negative bacilli. Absorption and excretion studies were followed by assays in the serum and urine by a microbiological turbidimetric method with *Shigella sonnei* as the test organism. A single dose of 1 g. usually produced a serum concentration of 10 μg. per ml., but considerable variations were observed. After oral administration chloramphenicol is rapidly absorbed, antibacterial activity appearing in the serum within 30 minutes ; but only a small fraction (from 4 to 10%) of the dose administered could be recovered from the urine, presumably because of the formation of inactive degradation products. In this way chloramphenicol differs from the other antibiotics, penicillin and streptomycin.

Chloramphenicol was tried in a wide range of clinical conditions. There were 93 cases of urinary-tract infection, due to a variety of organisms, 61 being due to a single organism and 32 to multiple organisms. Infections due to single organisms responded better than those caused by a mixed flora. A good clinical result was observed in 48 of the former and in 19 of the latter cases. Practically all the patients showed decrease in pyuria and bacilluria, even though the urine was not sterilized. In 63 cases the urine became sterile, and 52 of these were followed up for a period of at least one month, during which time there were 13 relapses. The daily dosage varied from 2 to 6 g. administered orally 4- to 6-hourly, and the duration of treatment varied from 5 to 7 days. Infections due to *Bacterium coli* also responded very well. There were 5 patients with primary atypical pneumonia, but the results obtained were equivocal. In 5 cases of pneumococcal pneumonia, with a daily

dosage of 3 g. for 5 to 8 days, there was a rapid symptomatic improvement and the temperature returned to normal within 48 hours, at which time pneumococci disappeared from the sputum. Chloramphenicol was used in the treatment of 4 patients with herpes zoster, but the course of the disease was not modified. Also, no marked beneficial effect was observed in any of 5 patients with chronic ulcerative colitis treated. Toxic side-effects were slight and were mainly related to the gastro-intestinal tract. The frequency of these side-reactions was related to the daily dosage; they occurred much more frequently in patients receiving 6 g. than in those receiving 2 g. daily. A bitter taste in the mouth was frequently noted and was sometimes accompanied by anorexia. Dryness of the mouth and throat, similar to that after atropine, was also observed. There was no evidence of renal damage or any changes in the blood picture. Drug fever was not observed, and there was only one case of skin rash. *R. Wien.*

Aureomycin in the Treatment of Experimental and Clinical Infections with *H. influenzae*, Type B. CHANDLER, C. A., and HODES, H. L. (1950). *Pediatrics*, **5**, 267.

The relative ease of administration of and absence of toxicity of aureomycin make the publication of reports of its effectiveness against bacterial invasion of various kinds a matter of intense interest. In a series of six cases, in children, of meningitis due to *Haemophilus influenzae*, Type B, full recovery was obtained with aureomycin therapy in all but one, the patient in this case being moribund on admission. Quicker results followed the combined use of aureomycin and sulphadiazine than of aureomycin alone. An average dosage of aureomycin for an infant was 50 mg. intravenously and 125 mg. by mouth immediately, followed by a daily dosage of 100 mg. intravenously for 6 days, and 500 to 750 mg. orally for 14 days. Experimentally, the effectiveness of aureomycin against *H. influenzae* infection in mice was equal to that of streptomycin and dihydrostreptomycin. *W. G. Wyllie.*

A Slide Culture Method for Streptomycin Sensitivity Testings. CUMMINGS, M. M., DRUMMOND, M. C., and SCHWARTZ, H. B. (1950). *Dis. Chest*, **17**, 202.

The slide culture method was compared with the solid culture technique in testing sensitivity of *Mycobacterium tuberculosis* to streptomycin. The Muller modification of the Pryce slide culture method, with "tween 80" albumin medium to which appropriate amounts of streptomycin were added, was employed. Results with this method could be read after 7 to 14 days' incubation. Specimens of sputum were examined before, during, and after streptomycin treatment. The specimen was divided, one-half being concentrated by the routine method with sodium hydroxide and seeded on Loewenstein–Jensen media containing 0, 1, 10, and 100 μg. of streptomycin and the other half being used for slide culture.

In 38% of 176 specimens positive cultures were obtained by both methods. The routine culture method revealed another 19.3% of positive specimens which gave negative results on slide culture; on the other hand, 3.5% were positive on slide culture and negative on routine culture. Only in 8 out of the 67 cultures positive by both methods was there disagreement in sensitivity readings, a higher degree of resistance being revealed by the slide culture.

Slide cultures, if satisfactory, gave a reading 2 weeks before the routine cultures were positive. In the case of sputum with scanty acid-fast bacilli the latter often fail to grow on slide culture.

E. Nassau.

Penicillin-resistant Staphylococci. Incidence in Relation to Length of Stay in Hospital. CAIRNS, H. J. F., and SUMMERS, G. A. C. (1950). *Lancet*, **1**, 446.

Penicillin sensitivity was studied of strains of *Staphylococcus pyogenes* iso-

lated from 326 in-patients, whose records showed how long they had been in hospital before the organism was isolated. The strains of *Staph. pyogenes* were divided into sensitive and resistant ones, according to their reaction on a penicillin gutter-plate containing 1 unit of penicillin per ml. of agar in the gutter. The patients were divided into five groups and the percentages of penicillin-resistant strains analysed separately for each group. Three groups of patients all had an infection, and the following results were obtained : of 70 cases of closed infection present on admission 19 yielded resistant strains and of 103 cases of open infection present on admission 43% were resistant to penicillin, whereas in 68 cases of infection arising in hospital the percentage of resistant strains was as high as 78. The remaining two groups consisted of 57 patients in whom the infection role of the staphylococcus was doubtful and in 46% of whom the staphylococcus was penicillin-resistant, and 28 patients without infection (the staphylococcus being isolated, for example, from a nasal swab) 61% of whom yielded a resistant strain.

Further analysis of the 103 cases of open infection showed that out of 51 cases from which the swab was taken within 1 day of admission 25% yielded resistant strains whereas 68% yielded resistant cultures when the swab was taken 8 or more days after admission. When these cases were analysed separately according to whether or not penicillin had been given before the swab was taken, it was shown that previous penicillin therapy did not increase the percentage of resistant strains.

[These results clearly emphasize the importance of hospital cross-infection in the production of penicillin-resistant staphylococcal infection. Since, however, 19% of cases of closed infection present on admission yielded penicillin-resistant staphylococci it seems rather an overstatement to say that "resistant strains would rarely emerge if cross-infection were avoided."]

Mary Barber.

BIOCHEMISTRY

Observations on the Physiologic Effects of Cortisone and ACTH in Man. SPRAGUE, R. G., POWER, M. H., MASON, H. L., ALBERT, A., MATHIESON, D. R., HENCH, P. S., KENDALL, E. C., SLOCUMB, C. H., and POLLEY, H. F. (1950). *Arch. intern. Med.*, **85**, 199.

The effects were studied, in a series of 33 patients, of the prolonged administration of 17-hydroxy-11-dehydrocorticosterone (cortisone) or pituitary adrenocorticotrophic hormone (A.C.T.H.). One of the patients also received 17-hydroxycorticosterone (compound F). Seventeen of the patients had rheumatoid arthritis and 5 rheumatoid arthritis and spondylitis ; 8 had acute rheumatic fever ; one had disseminated lupus erythematosus ; and one psoriasis and psoriatic arthritis. Among the changes produced by the administration of these hormones were increase in body weight, rounding of the facial contours, and the production of acne, hirsuties, keratitis pilaris, and cutaneous striae. Muscular weakness, amenorrhoea, and euphoria were sometimes found. The urinary excretion of corticosteroids rose above the normal level during administration of the hormones, while that of 17-ketosteroids fell rapidly at first, later becoming stabilized or rising again. There was no apparent relation between the effects produced by the hormones on the metabolism and the clinical response of the arthritis. There was an increased excretion of creatine and of uric acid in the urine and the production of a hypochloraemic hypopotassaemic alkalosis. The effect of the hormones on sodium and chloride balance was variable, but there tended to be a retention of both in the early stages and an increased excretion after prolonged administration. A negative nitrogen balance was produced, together with significant increases in urinary calcium and phosphorus excretion, and also an increase in faecal phosphorus. Conspicuous alterations in carbohydrate tolerance were not produced ; more striking

changes might be expected if the hormone were given to actual or potential diabetics.

Many of the manifestations of Cushing's syndrome were produced by protracted administration of cortisone, and there was evidence that cortisone is capable of depressing the function of the adrenal cortex in man as it does in the rat.

Geoffrey McComas.

The Value of Clinical Laboratory Tests. I. The Concentration of Cholesterol in the Serum of Patients with Disorders of the Thyroid Gland. FOOTE, J. B., and MERIVALE, W. H. H. (1949). *Guy's Hosp. Rep.*, **98**, 202.

In a study of the diagnostic value of estimation of the serum cholesterol level in disorders of the thyroid, groups of 56 cases of thyrotoxicosis, 24 of myxoedema, 17 of other thyroid diseases, and 50 normal adults were used, cholesterol estimation being carried out by the method of Bloor. The results in normal subjects ranged from 150 to 300 mg. per 100 ml. (mean 248.3 mg., S.D.±47.8). Statistical analysis of the results obtained in cases of thyrotoxicosis showed a significant lowering of the blood cholesterol level, but there was considerable overlapping in the range of concentrations found with that of the normal series, only 35% being outside the normal range. Untreated cases of myxoedema showed a significant rise in serum cholesterol level, with 65% of results outside the normal range, and a return to normal levels on treatment. The 17 patients with other thyroid conditions showed no significant alteration in their blood cholesterol level.

F. W. Chattaway.

The Excretion of 17-Ketosteroids in Idiopathic Hirsuitism. KOETS, P. (1949). *J. clin. Endocrinol.*, **9**, 795.

The urinary excretion of 17-ketosteroids in normal women fluctuates between 5 and 18 mg. per 24 hours. Where a masculinizing tumour is the cause of hirsutism the excretion is high or very high. From Stanford University School of Medicine, San Francisco, an investigation is reported on 17-ketosteroid excretion by 9 patients complaining of increased growth of facial and body hair for which no cause could be found. Their ages ranged between 21 and 30 years, the history from 2 to 4 years; 4 were single, 5 were or had been married, and none had children. Each week throughout the menstrual cycle 24-hour urine specimens were analysed for 17-ketosteroids by an adaptation of the method of Robbie and Gibson (*J. clin. Endocrinol.*, 1943, **3**, 200). Two normal women were used as controls.

The average excretion level was shown to be above normal although fluctuations to normal occurred; there was a cyclic variation, with the maximal excretion values at the approximate time of ovulation.

For comparison, daily excretion of ketosteroids was estimated in normal female rats. The results showed a marked variation of excretion with the maximum at mid-oestrus, a time which corresponds to ovulation in women. After ovariectomy the cyclic variation ceased and the excretion level fell below normal. After adrenalectomy the excretion was below measurable levels.

It is concluded that the increased ketosteroid excretion in idiopathic hirsutism is the result of a stimulation of the adrenal cortex by ovarian hormones. This sensitivity of the adrenal cortex is absent or imperceptibly small in normal women.

D. W. Higson.

Further Studies with the Salt Tolerance Test in Normal Individuals and in Patients with Adrenal Cortical Hyperfunction. SOFFER, L. J., GABRILOVE, J. L., and JACOBS, M. D. (1949). *J. clin. Invest.*, **28**, Part II, 1091.

The salt tolerance test (Soffer *et al.*, *J. clin. Invest.*, 1944, **23**, 51) was carried out on 22 normal subjects and on 15 patients with adrenal cortex hyperfunction. In 10 patients administration of deoxycortone acetate (DCA) resulted in a diuresis of sodium and chloride. After *x*-ray treat-

ment to the pituitary of one patient in whom there had previously been salt diuresis following DCA administration, salt retention was demonstrated. The blood sodium concentration was measured in 9 patients and was found to be considerably increased in 3 cases, in which the tolerance test showed salt retention, and normal in the others. Only in one out of the six cases in which the blood sodium concentration was normal was salt retention demonstrated. *A. Schweitzer.*

Electrophoretic Findings in Myelomatosis. SOULIER, J. P. (1950). *Sang*, **21**, 37.

This is a well-documented description of the electrophoretic findings in myelomatosis.

The conclusions are : (1) There is no invariable type of electrophoretic curve in myelomatosis. Four different electrophoretic types have been observed, and in 9 cases the curve was sufficiently characteristic to permit of a diagnosis. (2) There is no relation between the presence or absence of proteinuria and the electrophoretic curve in myelomatosis. In 5 cases, which did not belong to the same electrophoretic type, proteinuria was found. A distinction is made between the presence of thermosoluble protein of Bence-Jones, or globulin of small molecular weight, and non-thermosoluble proteinuria. (3) There is no relation between the clinical features, the presence or absence of proteinuria, and the histological type on the one hand and the electrophoretic characters of the serum on the other. *A. Piney.*

HAEMATOLOGY

Vitamin B_{12} and Folic Acid in Megaloblastic Anaemias of Pregnancy and the Puerperium. UNGLEY, C. C., and THOMPSON, R. B. (1950). *Brit. med. J.*, **1**, 919.

Six patients with megaloblastic bone marrow (having pernicious anaemia of pregnancy or the puerperium) were treated with vitamin B_{12}. They were given an injection of 65 to 80 μg. of the crystalline vitamin or red pigment concentrate, without haematopoietic or clinical responses until folic acid (2.5 to 20 mg. by mouth daily) was also given. Four also received a preliminary blood transfusion. There was no evidence of dietary deficiency in these patients. Marked haemolysis and reticulocytosis which were noted in one case ultimately ceased after treatment with folic acid, but additional liver extract was necessary to produce complete remission. *John F. Wilkinson.*

Vitamin B_{12} in Pernicious Anaemia : Parenteral Administration. UNGLEY, C. C. (1949). *Brit. med. J.*, **2**, 1370.

In this paper are recorded the results of treating 73 patients with pernicious anaemia by means of single injections of 1.25 to 160 μg. of vitamin B_{12}. A dose of 1.25 μg. had little or no effect, but 2.5 μg. produced a small response. A dose of 10 μg. produced on an average the "expected" response in respect of increases in reticulocytes and increases in red cells during a 15-day period. Larger doses produced greater increases. Twenty-one patients were studied for periods of 6–15 months, during which time they were given 10 μg. of vitamin B_{12} at 2-week intervals. In 18 of these patients the blood counts were maintained at satisfactory levels.

Variable Response to Vitamin B_{12} of Megaloblastic Anemia of Infancy. WOODRUFF, C. W., RIPY, H. W., PETERSON, J. C., and DARBY, W. J. (1949). *Pediatrics*, **4**, 723.

Three children aged 17, 9, and 12 months with manifestations of defective nutrition and a severe megaloblastic anaemia were treated with parenteral doses of 25, 25, and 30 μg. of vitamin B_{12} respectively. The first two cases responded satisfactorily. The third case did not respond and a megaloblastic marrow persisted. A satisfactory response, however, followed the administration of folic acid in daily doses of 5 mg.

Clinical Tests of a New Coumarin Substance. A Report to the Medical Research Council. BURT, C. C., WRIGHT, H. P., and KUBIK, M. (1949). *Brit. med. J.*, **2,** 1250.

The treatment is discussed of 126 patients suffering from various thrombotic diseases by means of a new coumarin product, bis-3 : 3′-(4-oxycoumarinyl)-ethyl acetate (B.O.E.A.). The plasma prothrombin level was reduced to under 50% of the normal within 36 hours in 100 of the patients, and returned to over 50% of normal within 36 hours of cessation of treatment. Toxic effects were slight.

The Use of ACTH in the Treatment of Acute and Subacute Leukemia. A Preliminary Note. DAMESHEK, W., SAUNDERS, R. H., and ZANNOS, L. (1950). *Bull. New Engl. med. Center,* **12,** 11.

This report is concerned with the response to ACTH of 5 children with acute or subacute lymphatic leukaemia. Remissions resulted, and in 4 children the blood and bone marrow became normal or almost normal. Remission was associated with an increased production of reticulocytes and platelets and a shrinkage of previously enlarged lymph nodes, liver, and spleen. The course in two adults with subacute myelocytic leukaemia and of an adult very ill with lymphatic leukaemia was not influenced.

The authors warn against optimism in the use of the drug.

Transfusion Reactions to a Plasma Constituent of Whole Blood. Their Pathogenesis and Treatment by Washed Red Blood Cell Transfusions. DAMESHEK, W., and NEBER, J. (1950). *Blood,* **5,** 129.

The authors describe the successful transfusion with saline-washed red cells of 11 patients who were apparently sensitive to some factor(s) in human plasma and who had developed reactions after the transfusion of whole blood. The patients were suffering from chronic haemolytic anaemia (6 cases), leukaemia (3 cases), and myelofibrosis and carcinomatosis (1 case of each). The reactions were characterized chiefly by rigors and fever and often a lack of red cell response, but haemolysis was not proved to be a cause.

The nature of the plasma factor was not determined ; the reactions did not seem to be due to the transfusion of iso-antibodies.

In several cases the therapeutic benefit of transfusing washed cells was striking and even life-saving.

Hereditary Nonspherocytic Hemolytic Anemia. CROSBY, W. H. (1950). *Blood,* **5,** 233.

Several members of a family suffered from chronic haemolytic anaemia and also brachyphalangia. The anaemia was normocytic ; there was no spherocytosis or increase in osmotic fragility. In the first patient studied porphobilinogen was found in the urine indicating an abnormal porphyrin metabolism. The disease was not cured by splenectomy. Transfusion studies clearly showed that the haemolysis was determined by a defect of the patient's own red cells.

The Haematological Effects of Nitrogen Mustard Therapy with Special Reference to the Cytology of the Sternal Bone Marrow. BROWN, A., and DAVIS, L. J. (1950). *Glasg. med. J.*, **31,** 93.

The authors have studied in detail the blood and bone marrows of 39 patients suffering from various reticuloses and bronchial carcinomata who had been treated with injections of nitrogen mustard. The total erythrocyte, leucocyte, and platelet counts were depressed in almost all the patients. The general reaction in the bone marrow was that of hypoplasia of all degrees down to almost complete aplasia. An increase in plasma cells and reticulum cells was an early sign. Evidence of regeneration was found two to three weeks after cessation of therapy, and during the fourth week the marrow became hyperplastic.

Blood and Bone Marrow Studies in Renal Disease. CALLEN, I. R., and LIMARZI, L. R. (1950). *Amer. J. clin. Path.*, **20,** 3.

In 44 patients with evidence of nitrogen retention due to renal disease, the severity of anaemia was related to the degree of nitrogen retention. In 80% of patients the bone marrow was hypercellular. A minor degree of hypoplasia of the erythroid tissue was observed only in patients having concentrations of non-protein nitrogen greater than 150 mg. per 100 ml. of plasma. Leucopoiesis and thrombopoiesis were not depressed.

Parahaemophilia (Owren), a New Type of Haemorrhagic Diathesis. FRANK, E., BILHAN, N., and EKREN, H. (1950). *Acta haematol. Basel,* **3,** 70.

The authors describe a patient suffering from a haemorrhagic diathesis due to a deficiency of Owren's factor V. Vitamin K had no effect on the prolonged prothrombin time, but the addition *in vitro* of prothrombin-free plasma or pure factor V restored the prothrombin time to normal. The patient's plasma added to haemophilic plasma corrected the deficiency in the latter, while prothrombin-free plasma from a haemophiliac corrected the defect of the patient.

Granulomatous Lesions in the Bone Marrow in Infectious Mononucleosis. A Comparison of the Changes in the Bone Marrow in Infectious Mononucleosis with those in Brucellosis, Tuberculosis, Sarcoidosis and Lymphatic Leukemia. HOVDE, R. F., and SUNDBERG, R. D. (1950). *Blood,* **5,** 209.

Granulomatous lesions closely resembling those found in the bone marrow in cases of brucellosis, and which were also not unlike the lesions of sarcoidosis and tuberculosis, were found in 11 out of a series of 23 cases of infectious mononucleosis studied by means of marrow biopsy.

MORBID ANATOMY AND HISTOLOGY

Cytohistologic Diagnosis of Material Aspirated from Stomach. Accuracy of Diagnosis of Cancer, Ulcer and Gastritis from Paraffin - embedded Washings. RICHARDSON, H. L., QUEEN, F. B., and BISHOP, F. H. (1949). *Amer. J. clin. Path.*, **19,** 328.

The deposit of gastric washings obtained after an alcohol–histamine meal were fixed in picro-formol, embedded, sectioned, and stained with haematoxylin-orange G-eosin. In normal stomachs squamous cells predominate, with some cubical or columnar epithelium in which small, deeply staining nuclei are present. In gastritis, large strands of mucosa infiltrated with leucocytes appear. In cancer the cells have large, deeply staining nuclei, usually containing prominent nucleoli ; the cytoplasm is scanty, vacuolated, with ill-defined boundaries. Ulcers yield a fibrin mass containing leucocytes.

Of 60 patients (27 with cancer, 24 with gastric ulcer, and 8 with gastritis) and 18 normal subjects examined, a correct diagnosis was obtained in 13 cases of cancer, 20 of ulcer, and 6 of gastritis and in 11 normal subjects. Two false-positive diagnoses of cancer in patients with ulcer or gastritis were made.

[This is an important paper, and should be read by all concerned with the diagnosis of these diseases.] *G. Discombe.*

Cytologic Changes in Bronchogenic Carcinoma Following Treatment with Nitrogen Mustard (Methyl-bis [β-Chloroethyl] Amine). GAENSLER, E. A., MCKAY, D. G., WARE, P. F., and LYNCH, J. P. (1948). *Arch. Path.*, **46,** 503.

Of 8 cases studied during treatment, 3 were of epidermoid carcinoma, 2 of squamous-cell carcinoma, and 3 of the "oat-cell" type. In the well-differentiated types the main changes produced by nitrogen mustard were giant-cell formation,

nuclear fragmentation, and a rather surprising increase in mitosis. On the other hand, in the oat-cell tumours large areas of necrosis and a decrease in mitosis were found. The maximum effect was observed in each case 5 to 8 days after treatment was started. Post-mortem studies showed great destruction of lymphocytes and haemopoietic tissue; also of the spermatogonia and their products.

Studies on Effusions. II. A Simple Technique for the Discovery of Cancer Cells in Neoplastic Exudates. CHU SHEN, S., and HOMBURGER, F. (1950). *Cancer*, **3**, 36.

The authors describe a simplified method of examining exudates for cancer cells which is more rapid, and possibly more reliable, than the Papanicolaou technique commonly employed. The centrifuged deposit from 20 ml. of defibrinated effusion fluid is resuspended in homologous or adsorbed serum and recentrifuged. From the top layer of this final concentrate, 3 to 6 smears are made and stained with Wright's stain in a phosphate buffer at *p*H 6.4.

They used this method in examining 37 samples of fluid from 26 patients with ascites, pleural effusion, or pericardial effusion. Of 20 cases in which there was a definite diagnosis of neoplasm, positive smears were obtained in 17, while of 10 of these cases studied by the Papanicolaou technique only 6 gave positive results. No false-positive results were obtained in 6 cases of non-cancerous effusion.

[This is a well illustrated paper and should be read in the original by anyone interested.] *A. Michael Davies.*

Carcinoma Cells in Sputum and Bronchial Secretions. A Study of 150 Consecutive Cases in which Results were Positive. WOOLNER, L. B., and MCDONALD, J. R. (1949). *Surg. Gynec. Obstet.*, **88**, 273.

A diagnosis of bronchogenic carcinoma on the basis of positive findings in the sputum or bronchial secretions was made in 150 out of a total of 1,600 cases. This diagnosis was confirmed by means of biopsy, surgical exploration, radiological examination, or necropsy in 145 cases. Positive smear reports were proved to be false in 3 cases, and in 2 a final diagnosis was not established. *G. B. Forbes.*

The Malignancy of Cancer at Different Ages: A Histological Study. LEES, J. C., and PARK, W. W. (1949). *Brit. J. Cancer*, **3**, 186.

There has been a general impression among clinicians that the malignancy of cancer is greater in relatively young people. The authors analysed recent literature containing statistical data and did not find in it much support for the belief. They then went through the records of the Laboratory of the Royal College of Physicians of Edinburgh and obtained a random sample of microscopical preparations from subjects in three age groups and for seven sites. The following characteristics were chosen: degree of epidermoid or glanduliform differentiation; degree of fibrosis; cell size and polymorphism; amount of "colloid" formation; mitotic frequency; general impression of malignancy. Each feature was assigned to one of four grades in increasing expression. The resultant contingency tables were analysed (the total number in each age group was 105) and, with the very doubtful exception of carcinoma of the lung, no significant differences between the age groups were revealed. *Major Greenwood.*

Meningeal Tumours with Extracerebral Metastases. CHRISTENSEN, E., KLÆR, W., and WINBLAD, S. (1949). *Brit. J. Cancer*, **3**, 485.

A report of two cases, in males aged 38 and 40 years respectively, in which the operative removal of a meningioma from the Rolandic region was followed by metastasis to remote organs.

Ependymonas and Choroid Plexus Papillomas. RINGERTZ, N., and REYMOND, A. (1949). *J. Neuropath. exp. Neurol.*, **8**, 355.

An account of 72 examples of ependymona and 19 of chloroid plexus papilloma, including their pathology and postoperative course. In general their features accord with previous descriptions of these types

Carcinoma of the Parathyroid Gland with Hyperparathyroidism. DE WESSELOW, O. L. V., and DE WARDENER, H. E. (1949). *Lancet*, **1**, 820.

The authors refer to 3 cases, recorded in the literature, of parathyroid hyperfunction with evidence of clinical and histological malignancy and give clinical details of one further case. The patient was a woman of 35 who was first admitted to hospital in 1942 with classical signs and symptoms of hyperparathyroidism. A parathyroid tumour was removed on no fewer than 5 separate occasions between this date and 1948, and two courses of *x*-ray therapy were given. Symptomatic relief and lowering of the serum calcium level followed these procedures. In most of the relapses there was a sudden onset of symptoms and an associated cardiac failure, due probably to the high serum calcium level. Histological section of the tumour removed at the fifth operation showed a low degree of malignancy.

Geoffrey McComas.

Fibrosing Adenomatosis of the Breast. HELLER, E. L., and FLEMING, J. C. (1950). *Amer. J. clin. Path.*, **20**, 141.

This work, reported from the University of Pittsburgh and the Presbyterian and Women's Hospitals, Pittsburgh, is based upon 15 cases of fibrosing adenomatosis of the breast in women, forming 2% of the breast lesions examined in the pathology departments over a period of 5 years. The age incidence of the disease is 18 to 45 years (average 34 years).

Patients have a single, painless, circumscribed, firm, mobile mass from 0.5 to 5.0 cm. in diameter, sharply demarcated, but not encapsulated. The cut surface is usually solid and fibrous with a delicate granularity, and often contains a few small cysts 1 to 3 mm. in diameter. The lesion lacks the small yellow foci and the glistening translucency of the mammary carcinoma. In stage I there is hyperplasia of the ductules, which are embedded in a dense, collagenous, acellular band replacing the loose specialized, peri-acinar connective tissue. In stage II the deposition of collagen has completely replaced the peri-acinar connective tissue and thus caused compression and distortion of the ductules. At first sight the histological picture may be one resembling that of malignant infiltration, though cytologically there is no evidence of malignancy. In stage III intralobular fibrosis has completely destroyed the lobular structure; distorted narrow cords of acinar epithelium, 2 to 3 cells wide, lie in a disordered arrangement in an abundant collagenous matrix. The general resemblance to carcinoma may be striking, but the cytological evidence of malignancy is lacking.

The authors suggest that the condition is a variant of benign cystic disease of the breast in which the proliferation of the basement membrane of the ductule, rather than of the epithelium, predominates.

There has been no evidence of recurrence during the follow-up period. Local excision and, occasionally, local mastectomy is the treatment of choice.

Peter Harvey.

Sclerosing Adenosis. URBAN, J. A., and ADAIR, F. E. (1949). *Cancer*, **2**, 625.

In this paper a detailed description is given of the clinical picture, gross appearance, and morbid histology in 34 cases of a condition described as "sclerosing adenosis" (a form of "chronic mastitis"). The difficulty in differentiating the lesion from cancer resides first in the fact that the extreme degree of fibrosis gives to the gross specimen a feel very much like that of cancer, and histologically the ducts are crammed with epithelial cells. Nowhere,

however, is there true transgression of epithelial cells into the surrounding stroma, and in most cases an alveolar pattern, or at least spaces between the epithelial cells, are discernible under the microscope.

The Septic Abortion Kidney. WYATT, J. P., and GOLDENBERG, H. (1950). *Amer. J. Obstet. Gynec.*, **59**, 337.

The authors describe (under the name of "septic-abortion kidney") 3 fatal cases of lower nephron nephrosis after septic abortion.

The authors contrast lower nephron nephrosis with the rarer (though more frequently reported) symmetrical cortical necrosis, and an attempt is made to explain these two apparently diverse lesions in the light of Trueta's recent work on the renal circulation. The "medullary shunt" is believed to occur in cases of crush syndrome, but presumably a limited circulation, sufficient for nutrition but not for glomerular function, continues through the cortex, except in extreme cases, in which the restriction of the cortical circulation produces symmetrical cortical necrosis. On the other hand, the theory of tubular blockage by haematin casts has not been disproved, and the true answer may be that there is an interplay between the two factors of anoxia and haematin precipitation. But, whatever its aetiology, the condition should be suspected in any case of induced abortion with oliguria.

The Role of Lipid Deposition in Renal Arteriolar Sclerosis. WILENS, S. L., and ELSTER, S. K. (1950). *Amer. J. med. Sci.*, **219**, 183.

In 140 specimens obtained at necropsy, the renal arterioles of normal individuals were found to contain minute lipid deposits, the amount becoming increased in the later age groups. The distribution of lipid in the arteriolar wall was variable, usually occurring throughout the media, and bore no relationship to the presence of lipid in other structures such as glomeruli or tubules. In primary hypertension and diabetes the lipid was considerably increased, especially in those vessels which showed hyaline degeneration. Although a contrast is drawn between the lesions of atherosclerosis and of arteriolar lipidosis, it is concluded that in these two conditions lipid deposition results from variations in blood pressure in different sets of blood vessels.

Distribution of the Lymphatics of the Human Kidney as Shown in a Case of Carcinomatous Permeation. RAWSON, A. J. (1949). *Arch. Path.*, **47**, 283.

The distribution and arrangement of the lymphatic channels were studied in a kidney obtained at necropsy in a case of extensive carcinoma. Microscopical examination of the kidney showed that the lymphatic vessels were clearly visible and distended by solid cords of tumour cells.

One system of lymphatic channels began as minute blind-ending vessels in close contact with Bowman's capsules. No lymphatic vessels were seen round the afferent or efferent arterioles of the glomeruli and none appeared to penetrate through Bowman's capsule. The interlobular capillaries were also free from any lymphatic networks. The small lymphatic vessels beginning near Bowman's capsules gradually enlarged and formed nets round both the arterial and venous vessels of the cortex. Particularly dense networks were observed round the large thin-walled venous channels or "sinuses" lying in the outer half of the cortex. These lymphatic vessels round the blood vessels follow successively the interlobular, arcuate, and interlobar arteries and veins, leaving the kidney at the hilus to terminate in the nodes on either side of the aorta. Small blind-ending lymphatic capillaries also originated beneath the mucosa of the papilla, ascended in more or less straight lines through the medulla, and emptied into the larger lymphatic channels round the arcuate arteries and veins. The lymphatic trunks round the arcuate vessels therefore receive lymph from both medulla and cortex.

Histopathologic Observations in a Fatal Case of Q Fever. PERRIN, T. L. (1949). *Arch. Path.*, **67**, 361.

In this paper are described the histological findings in a previously recorded case. A 43-year-old cattle-worker died after a 15-day illness characterized by fever, cough, nasal congestion, headaches, and mental confusion. Necropsy revealed a diffuse consolidation of lungs, microscopy showing an essentially mononuclear exudate, with congestion and haemorrhages, but neutrophil granulocytes were not infrequent and there was a fibrin network. No rickettsiae or other microorganisms could be found. Focal hypoplasia was seen in the sternal marrow. In the myocardium there was fairly extensive ischaemic fibrosis due to occlusion of a major coronary vessel. No significant change was found in other organs. It is concluded that the lung changes were essentially due to the Q-fever virus and possibly also the marrow hypoplasia ; however, the cardiac condition certainly seems to have had an unfavourable influence in a disease usually benign in the healthy adult.

Diffuse Cystic Lungs of Granulomatous Origin. A Histological Study of Six Cases. CUNNINGHAM, G. J., and PARKINSON, T. (1950). *Thorax*, **5**, 43.

Detailed histological studies on 6 cases of honeycomb lungs are reported, and brief clinical records of the patients are given. All cases showed a granulomatous process in the walls of the cysts and in the intercystic spaces. In the acute phase this process was characterized by a highly cellular histiocytic response, and in the chronic phase by widespread fibrosis. In the intermediate stages, foamy macrophages were present in large numbers, sometimes being the predominant cell. It is suggested that the cystic spaces are produced by weakening of the walls of the smaller bronchioles as a result of granulomatous infiltration. In the present material the distinction between cysts of alveolar and bronchiolar origin is impossible. The nature of the granulomatous process is discussed. [Authors' summary.]

Diagnostic Value of Histologic Lesions of Striated Muscle in Rheumatoid Arthritis. SOKOLOFF, L., WILENS, S. L., BUNIM, J. J., and MCEWEN, C. (1950). *Amer. J. med. Sci.*, **219**, 174.

Biopsy or necropsy specimens or muscle from the deltoid and gastrocnemius were examined by serial section in 57 cases of rheumatoid arthritis, 10 of ankylosing spondylitis, 21 of acute rheumatism, 101 of various other conditions, some involving the joints, and in 13 healthy volunteers. Focal cellular lesions similar to those described by other workers were found in 56% of the cases of rheumatoid arthritis, but also in 3 of the normal controls and in 25% of the non-rheumatoid group as a whole. Various other laboratory investigations were performed, such as determination of the erythrocyte sedimentation rate and estimation of the serum content of streptococcal agglutinins and agglutin of sensitized sheep erythrocytes. None of these threw any light on the mechanism of production of the histological lesions, and the authors conclude that muscle biopsy is of little value as a diagnostic procedure in rheumatoid arthritis. *G. J. Cunningham.*

Correction

DR. LEVIN writes: I must apologize for an error, which was in our typescript and was no fault of the printers.

In the paragraph headed "Method" of "A Simple Visual Turbidimetric Estimation of Serum Gamma Globulin" (*J. clin. Path.*, **3**, 284) the first line should read: "Serum, 0.1 ml., is added to 2.4 ml. of saline ammonium sulphate solution" . . . instead of "Serum, 0.1 ml., is added to 4.9 ml. of saline ammonium sulphate solution."

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